

Escalation of animal rights war

Organizations that exist to promote the welfare of animals must dissociate themselves from the actions of those claiming to safeguard animal rights by using violence against people.

PROTESTS at the use of animals in research have taken on a new and awesome character in Britain with the attempted murder of two British veterinary scientists by the time-honoured terrorist technique of the pre-planted car-bomb. Both of those concerned escaped, one only just, but the Bristol bomb seriously injured a one-year-old child being carried along a sidewalk in a push-chair. No doubt those who planted the bombs will have learned from their mistakes, as from the occasion last year when a bomb was exploded in a common room at the University of Bristol, and will succeed in killing somebody. Further mayhem will no doubt follow.

The research community will rightly be alarmed at these developments, which have two objectives: to win publicity and to intimidate people working in research with animals (known as 'vivisectors' to the extremists). The first need is that everything should be done to identify those responsible for the crimes and to put them on trial. The Defence Research Society has taken the practical step of offering a reward of £10,000 for information leading to those responsible, but past experience is not encouraging. Small bands of people united only by an excess of zeal in a narrow cause are unlikely to be tempted by such offers. The professional police will similarly be confronted by the usual problem of finding a needle in a haystack.

Action

That is why the intellectual community in Britain and elsewhere must act more vigorously in its own defence. There are several steps that can be taken, of which the chief is to demand of all the organizations that exist with the declared objectives of safeguarding the interests of animals that they should declare explicitly where they stand on violence towards people. And it will not suffice that the chairmen and chairwomen of British organizations such as the Royal Society for the Prevention of Cruelty to Animals and the Anti-Vivisection League should utter placatory statements on behalf of all their members. These people should also undertake that it will be a test of continuing membership in their organizations that members and would-be members should declare that they will take no part in acts of violence against human beings. Even such undertakings would not be fully effective; people, after all, can lie. But at least they would distinguish the organizations entitled to a continuing

voice in the dialogue with the research community about the rights of animals in research from the organizations that deserve no say.

Network

There is an even darker issue to be taken up. Last year's break-in at an INSERM laboratory at Lyons in France followed close on the heels of the appearance of a related research report in *Nature*. Somebody had read the research report and tipped off the raiders. It would make good sense that groups bent on causing mayhem in animal laboratories should have established a network of some kind for the exchange of information internationally. There is ample evidence that just such a network is in being. Nobody can blame the animal welfare organizations for that, but their own interests require that they should be seen not to contribute, even inadvertently, to its success.

That is where the rub comes. Shock-horror pictures, even when faked, generate membership applications and funds. Horror stories often serve as well, even when they consist of packs of lies. The best-intentioned organizations are guilty of exaggeration of this kind. One does not need to be a Freudian to accept that the image of an anaesthetized mammal of any species is a model for one's own death. The paradox underlying the animal rights movement is that the passions occasioned by such threats are projected (as the psychoanalysts would say) back on to the animals. "Let us kill some people, so that the animals may be free", is how the argument goes. The claptrap of the philosophy of 'speciesism' (or of the failure of the gene to be unselfish) lends spurious support. But fund-raising propaganda, however effective, has no place in national dialogue if it is false. Animal rights groups seeking a continuing dialogue with the research community should also give that up.

What else would be left for them? Plenty. The images of animals under anaesthesia that grate on the unconsciousness of would-be members also move the feelings of those who work in laboratories. How could it be otherwise? The difference is that the would-be members blame the laboratory people and the laboratory people (with a few notable exceptions) believe they act in the interests of other people which — magnificently — they do. That conflict of interest, which is pragmatic and not philosophical, has been amply defined. The no-man's-land is



movable, and could be almost anywhere. But the animal rights movement will forfeit its say on the issue if it cannot shake off its extremists. Then it might find itself democratically in the hands of those with heart disease or stomach ulcers, who may have fewer doubts about the necessity of animals in research. □

Frankness needed

The British public needs more information about the problems of bovine spongiform encephalopathy.

PROFESSOR Richard Lacey, a microbiologist from the University of Leeds, was given a rough ride last week by the House of Commons committee inquiring into the occurrence of bovine spongiform encephalopathy (BSE) among British cattle. Lacey's interest was to suggest that the British government is mistaken in stating categorically that the agent responsible for BSE poses no threat of any kind to those who may eat British beef. When even the origin of the epidemic is uncertain and when so little is known of its mechanism, Lacey must be right in that. But in declaring that the damage that will eventually be done to people lies somewhere between zero and the "worst case" in which "a whole generation" will be lost to some human analogue of BSE, perhaps a syndrome indistinguishable from Creutzfeldt-Jakob disease, he went too far.

The first inquiry more than a year ago by a committee under Sir Richard Southwood identified BSE as the bovine analogue of scrapie in sheep and offered the hypothesis that it had arisen from the practice of feeding abattoir offal to cattle as a protein source. The hypothesis, not yet confirmed, is the basis of current British efforts at control, principally the destruction of diseased animals and the removal of brain, spinal cord and thymus from all apparently healthy cattle sent for slaughter. Some European governments have also won from the European Communities (see *Nature* **345**, 566; 14 June 1990) the right to refuse the import of carcasses from British herds in which cases of infection have been recorded.

Analogy

Nobody disputes the analogy between BSE and scrapie, implying that the agent of the disease is an entity called a prion, a small protein molecule perhaps associated with lipid, which evokes no worthwhile response from mammalian immune systems. Somehow, it appears, prions are replicated in infected cells (chiefly neurons), perhaps by blocking normal metabolism, perhaps by acting as genetic control elements or, perhaps, by some quite different mechanism. For the time being, nobody knows. The most urgent need is for understanding. The British Ministry of Agriculture, Fisheries and Food has commissioned some research, but should now be thinking of deploying the most sophisticated techniques of molecular biology in the search for an understanding of scrapie and of BSE.

Meanwhile, the Laceys of this world should be trying to fit numbers to the few known features of these diseases. So far, close on 10,000 cattle have been afflicted with BSE, a fraction of 1 per cent of the British cattle herd, but the cases are widely spread. On the hypothesis that the effective agent is derived from that of scrapie, does the pattern imply one interspecific transfer (or a few of them) early in the practice of feeding sheep offal to cattle (begun in the 1960s), followed by some cryptic method of spread through the cattle herd? Or is there no molecular barrier to transmission, in which case the risk that an animal eating infected feed will be affected by disease must be small? The British agriculture ministry has also dug into its pocket in this epidemiological cause, but could dig deeper.

Assertions

The essence of Lacey's case is that, if cattle can contract BSE by eating offal from sheep with scrapie, why should not people contract something like it by eating the flesh of affected cattle? The question is fair, but especially with the restrictions on the use of offal now in force, the sporadic occurrence of BSE among cattle argues that the chance of damage to people who eat beef is at most small, and is probably very small (and may be zero). Talk of the "loss" of whole generations (of people), implying both ready infection and some method of horizontal transmission, is inappropriate. But so too is the assertion that the scanty evidence now to hand rules out transmission, however improbably, of something like BSE to people.

The British government would be wise to find some way of making plain something akin to this in public. Everybody will sympathize with its difficulty — the danger of doing great economic damage to British farmers by language that may be misrepresented in the commercial markets on which beef is sold for the sake of avoiding a risk that is eventually shown to be negligible even in the eyes of those who eat beef. There are precedents in the international nuclear industry, which has been much damaged by official assurances that the risks are "negligible". The British ministry's best strategy in these circumstances is *glasnost*. The Committee for the Public Understanding of Science (set up by the British Association, the Royal Society and the Royal Institution) has made a start. Today the chairman of the spongiform encephalopathy advisory committee, Dr. D. A. J. Tyrrell, gives a public lecture entitled "What should we think about BSE?" The agriculture ministry should be joining in, satiating the British public with information about the incidence of BSE (in the knowledge that if the Southwood hypothesis is correct and its control strategy is effective, the incidence should decline in a few years) and explaining in great detail what it hopes to learn from the research it has commissioned. This is not to suggest that ministry officials have been secretive so far, but that even their political masters would benefit if they could accept that Lacey's question is fair — and then quarrel with him about the numbers. □

Revenge of the taxman on grant recipients

■ Grants treated as 'foreign funds'

■ Japanese scientists looking for loopholes

Tokyo

WHEN Japanese scientists won grants under Japan's international Human Frontier Science Program earlier this year, they never imagined that they might find themselves regarded as in receipt of 'foreign funds' and due for a visit from the taxman. But Japan's labyrinthine bureaucracy and interministerial rivalries seem likely to ensure that recipients will have to give half their grants back to the Japanese government.

The problem arises because the Frontiers programme is based in Strasbourg, which means that its grants are considered to come from a foreign country, even though almost all the money was supplied by Japan in the first place. Under university regulations laid down by the Ministry of Education, Culture and Science (MESC), which has never much liked the frontiers programme and is the least international of the organizations supporting research, there is no mechanism for universities to receive grants from overseas.

The Human Frontier Science Program was established as a foundation in Strasbourg last year by the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency (STA) in consultation with scientists and government representatives of the seven summit nations (Japan, the United States, Canada, the United Kingdom, France, Italy and West Germany) and the European Communities (EC). The foundation awarded its first grants and fellowships for research on the brain and molecular biology in April and many of the recipients were Japanese (see *Nature* 344, 479; 5 April 1990).

Unless MESC regulations are rewritten, the only way for Japanese university researchers to receive Frontier grants is to take them into their personal bank accounts and then 'donate' the money to their university. Even though the money is donated for scientific research, under Japanese tax laws laid down by the Ministry of Finance 75 per cent of the grant will be considered 'personal income' of the scientist and so liable for income tax.

Nobuhiro Go of the Department of Chemistry of Kyoto University, who is expecting to receive \$96,000 a year for 3 years out of a grant of \$400,000 a year awarded to principal scientist Fuyuhiko Inagaki of Tokyo Metropolitan Institute of Medical Science, reckons that if he accepts the grant this way, 40 to 50 per

cent of it will be given back to the taxman because it will be added to his own personal income and taxed at the highest rate.

Toichi Sakata, director of STA's Frontier office, says his colleagues are working "intensely" on the problem in consultation with MESC and the researchers involved. He thinks they can solve the problem but he admits that they have "no experience of such a situation".

MESC cannot be expected to fall over backwards to help find a solution — it has always been very cool towards the Frontier programme, seeing it as an attempt by STA and MITI to encroach on its own territory. And the Ministry of Finance appears in no hurry to change its tax rules.

Go thinks he may have found a personal solution. It so happens that Inagaki, the principal scientist of Go's Frontier grant, belongs to an institute that is classified as a private foundation. Go thinks it may be possible for Inagaki's institute to receive Go's share of the grant and pass it on to Kyoto University because under MESC regulations there is comparatively little



difficulty in a university receiving money from a domestic private foundation.

Go emphasizes that this solution will not apply to his five other colleagues at Kyoto University who have won Frontier grants. And he says that what is needed is a general solution that will open up and "internationalize" Japan's universities. But he fears that STA, MESC and university officials are going to try to solve the problem at the individual level.

Mitsuhiro Yanagida, another Frontier recipient at Kyoto University, sees the whole affair as "ironic black humor". He suspects that Japanese government officials were so busy negotiating with foreign government officials and scientists that they forgot to think about such domestic problems. He says he is thinking of accepting the money as "personal income" and defying the taxman to come and tax him.

David Swinbanks

BRAZILIAN UNIVERSITY REFORM —

Further success for excess — more places and more courses

São Paulo

LAST week, the Brazilian federal universities emerged victorious from a meeting with the Minister of Education, Carlos Chiarelli. They were supposed to volunteer plans to dismiss 42,000 employees but they managed instead to persuade the minister that they could save money by cutting salary bonuses, providing more places for students and opening more courses.

The university dismissals were part of a plan under which the federal government should have struck more than a third of a million employees from its bloated payroll by this week. The Brazilian federal government has about 1.7 million civil servants (no one has an exact figure). President Fernando Collor de Mello's basic election campaign promise was to rid Brazil of inefficient public officials, especially those nicknamed 'maharajahs' who have managed to amass big salaries by adding on all kinds of extra bonuses. But it now seems that Collor will not quickly win success with this part of his economic reforms.

Public servants have a bad image in Brazil. Recently, the archives of the federal censorship bureau were opened and composers of pop songs were able to find out why some of their songs were banned. One was 'Va Trabalhar, Vagabundo' ('Go to work, vagabond'), by Chico Buarque de Holanda, a famous singer and songwriter. The censor had said that the song might be offensive to public servants.

The problem for Brazil's federal universities is that they are a mixed bag, a sea of inefficiency with a few islands of competence. Statistics of the Ministry of Education, which funds the 36 universities plus 15 other isolated faculties, show that on average, only 16 per cent of faculty members have PhDs, compared with about 80 per cent for US universities. At Brazil's most respected institution, the University of São Paulo, which is supported by the state of São Paulo, about 65 per cent of faculty members hold PhDs.

University rectors cannot complain that they are understaffed. There is an average of eight students per faculty member. In the University of São Paulo, the rate is 10 students to one teacher; and in US universities the usual ratio is 15.

But dismissing university employees is complicated and difficult because of the maze of laws and regulations that govern tenured posts.

Following the meeting between the rectors and the university, it seems that the result will be basically the same as with the overall reform — the status quo will be maintained, and the impetus for change will fizzle out.

Ricardo Bonalume

Anyone speak Korean?

Washington

AFTER a two-week search for Asian partners for the Superconducting Super Collider (SSC), US Department of Energy (DOE) officials returned last week with a 'surprise' letter of support from South Korea. But although DOE held a press conference at which deputy energy secretary W. Henson Moore interpreted the letter as Korea's intention to become a partner in the \$8,000 million accelerator project, it remains unclear what, if anything, Korean officials have actually promised.

DOE released a photograph showing US energy secretary James Watkins and Korean minister of science and technology Kunmo Chung exchanging a letter that can be seen to be written in Korean. On the back of the picture, the letter is identified as a formal acceptance by Korean President Tae Woo Roh of President George Bush's offer to become a partner in the SSC. When pressed, however, DOE officials admitted that they are not entirely sure what the document signifies. "We take it to mean that the Korean

government is very interested in joining the project", Moore said.

A DOE spokesman says that the confusion arose because the Koreans had not supplied an English translation of the letter, and the initial US attempt at translation apparently missed some nuances.

On reflection, DOE has reinterpreted the letter to mean that Roh is considering the offer and will have an answer soon. Unfortunately, DOE refuses to release the text of the letter in either English or Korean, and the photograph is just a bit too indistinct for it to be possible to read more than scattered words.

Moore said that he had left the Koreans with a list of "appropriate ways they could discharge their partnership responsibilities". Although he did not specify what those ways were, he said that cash, magnets and other technology were all options (see *Nature* 345, 563; 14 June 1990).

Unexpectedly, Korea responded by giving DOE its own list of contributions it thought were appropriate. Sources who

HUBBLE SPACE TELESCOPE

met with Chung say that although the Korean government is indeed interested in collaborating on the SSC, the two countries still differ widely on terms. The United States wants money, they say, but Korea would rather provide manpower, skills and knowhow.

If Korea does join the SSC, it would be the project's first international partner. Given that the SSC is now expected to cost about twice the original estimate, DOE no longer considers India, which had earlier agreed to participate, bound to its promise.

Japan, DOE's chief target in the recent quest for partners, made few gestures of encouragement. But Moore denied the suggestion that the Japanese government had gone to great lengths to dissuade DOE from visiting, out of reluctance to being put in the position of having to say no. An advance DOE team had asked Japan whether Moore's team should come to make the formal request, Moore says, and Japan gave the green light. "I doubt that the Japanese are going to say that they have no intention of working together", he said. **G. Christopher Anderson**

ACOST

Strategic reviews to be published

London

RESPONDING to pressure from the House of Lords Science and Technology Committee, British prime minister Margaret Thatcher has decided that the government's Advisory Council for Science and Technology (ACOST) will publish a strategic review of British science and technology every three years.

Mrs Thatcher had earlier insisted that ACOST's advice should remain confidential. But Lord Flowers, chairman of the Lords committee, wrote to the prime minister on 6 March, arguing that the annual review of government funded research and development was inadequate to assess the progress and priorities of British science. Mrs Thatcher's reply says the new ACOST reviews will identify future issues to be examined in the public and private sectors.

The first review will be the responsibility of Sir Robin Nicholson, an industrialist who takes over as chairman of ACOST from Lord Tombs for a three-year term beginning in July.

ACOST was established in 1987 at the suggestion of the House of Lords Science and Technology Committee, to advise the government on priorities for British science, including the application of research and international collaboration.

Peter Aldhous

Light at the end of the tunnel

Washington

THE persistent vibration problems that have plagued the Hubble Space Telescope since its launch last month may soon be solved, National Aeronautics and Space Administration (NASA) officials said last week. A planned modification in the on-board control software is expected to correct the problem and allow the telescope to operate within design specifications in as little as a month, although a full schedule of scientific observations may not begin until late in the year.

NASA engineers have traced the vibration problem to thermal expansion of the telescope's large solar arrays. When Hubble moves from night to day and vice versa, the panels heat up unevenly and begin to sway. The 6 arc-second oscillations last for three to five minutes after the transition, during which time fine-track observations are difficult, says project manager Fred Woitlik.

Because the telescope could be tested under the force of gravity only on the ground or with a computer simulator, the vibrations had not been fully anticipated. "The array gets a lot of heat quickly, and sees a thermal differential that is a lot larger than showed up in [pre-flight] analysis", Woitlik says.

On-board control software was designed to dampen less severe mechanical oscillations, and tends to "overflow its stacks" when confronted with vibrations as large as those generated by the heated solar arrays, he says.

Although correcting the software simply requires changing several parameters to make the control loop "stiffer", NASA must thoroughly test the modifications to ensure that they do not have unexpected — and potentially disastrous — side effects. Testing is expected to take another three to five weeks, Woitlik says. Once approved, the new software will be uploaded to Hubble.

The only other serious problems is in a radiation-sensitive portion of the guidance electronics. NASA engineers knew that the fine-lock system was less 'hardened' to radiation than the rest of the Hubble circuitry, but they decided to take the chance that it would not prove to be a problem. Once Hubble was in space, however, radiation in the South Atlantic Anomaly, the area of orbit where the Van Allen radiation belt comes closest to Earth, played havoc with the fine-lock electronics. The system would often reset itself when Hubble passed through the anomaly, requiring mission controllers to turn the system off and on again manually.

NASA's solution to the problem, while inelegant, seems to work. New software has been written to reset the fine-lock electronics five times a second throughout its orbit. Simulations indicate that Hubble's performance is not degraded by the manoeuvre. Indeed, when all the software changes are in place, NASA engineers expect to bring the telescope to within its original 0.007 arc-second stability goal.

G. Christopher Anderson

Rotten to the core

New Delhi

THE way research is being conducted in Indian universities should be a cause of grave concern to scientists and planners alike, according to a new study from the Council of Scientific and Industrial Research. The study says the malaise is so deep rooted that it would take at least a decade to rejuvenate university research.

The study, entitled *Quality, Character and Efficiency of Scientific Research in Universities and Indian Institutes of Technology*, was directed by Professor Rais Ahmed, former chairman of the University Grants Commission. It is based on questionnaires given to supervisors and scholars in 27 selected institutions, including the five Indian Institutes of Technology, and six central and 16 state universities. In all, 824 professors and readers and 1,740 scholars responded. Analysis of the data reveals poor academic performance and frequent complaints of malpractice and unfair procedures.

The study shows that more than half of top-ranking students do not go into research. Those who do tend to come from poor backgrounds and lack motivation or enthusiasm. Eighty per cent of scholars have knowledge confined to their subject and half are "unable to read scientific literature or write a report".

There is very little guidance or supervision of university research, says the study. Supervision is on a casual basis and, in 80 per cent of universities, meetings between scholar and supervisor take place less than once a year. Forty per cent of supervisors do not actively participate in research or check experimental data.

The research environment in the universities is sadly undermined by prejudice and malpractice. The study says that scholars are assessed not on merit but on the basis of factors such as sex, caste, religion or the part of the country they come from. "The sum total of prejudice must be acting like poison to the atmosphere in the lab", the report says. Academic research is also tarnished by malpractice such as the manipulation and forging of data or the award of degrees in spite of critical reports from examiners. Between 30 and 50 per cent of scholars said they were aware of malpractice. "These dishonourable practices are more common than one would have thought", the report says.

Academic values are also found to be lacking. Internal examinations fail properly to discriminate between good, indifferent and bad scholars. At least 40 per cent of supervisors think fit to recommend the award of doctorates to students whose theses were based on "unsound concepts or wrong methodology". Universities also suffer from too much inbreeding. As

many as 90 per cent of faculty vacancies are filled from within.

One reason for the dismal atmosphere in the universities is poor funding, according to the report. The universities are the largest single research sector and employ 30 per cent of India's scientists and half of its PhDs but receive only six per cent of the national research and development budget. The universities have been left to vegetate and their work "is not closely examined, analysed or planned" by any

MARS MISSION

1-800-GOTOMARS

Washington

REMEMBER nuclear rockets? Anti-matter drives, solar sails, electromagnetic propulsion systems? Twenty years ago, at the height of the Apollo era, far-fetched plans for interplanetary travel were all the rage. The US National Aeronautics and Space Administration (NASA) is hoping that those kinds of ideas are still around. Last month it announced a \$4.5 million programme to scour the country for better ways to reach Mars — better, hopefully, than the \$500,000 million plan NASA put together last year after President George Bush backed Mars as the space agency's next goal.

Last week, Richard Truly, administrator of NASA, sent out 3,200 'personal' letters to presidents of universities, heads



of research institutes and individual scientists in the United States, asking them to propose new "architectures", propulsion systems or materials for the Mars Mission. Truly does not want to hear from just the usual scientists; his offer is open to everyone from backyard inventors to teenage science wizards.

Operators at a 24-hour, seven-days-a-week toll-free number (1-800-677-7796 in the United States) are waiting to send out packages to help budding space planners send in their ideas.

Truly has appointed Thomas Stafford, a former astronaut, to lead a 'synthesis group' which will review incoming ideas and make recommendations as to which should be pursued further. One idea

internal or external forum.

Universities urgently need funds, but money alone cannot change them without a radically different educational pattern, says the study. It is suggested that eight regional institutions be established with a new pattern of undergraduate and post-graduate education for which students will be selected by a national talent search. Universities need also to be persuaded to admit only research scholars screened through a nationally administered test. The whole system needs to be rebuilt, small cosmetic changes will not help, the study concludes.

K. S. Jayaraman

Stafford's panel already has on its table is for an inflatable spacecraft designed by Lowell Wood, the scientist at Lawrence Livermore National Laboratory who invented 'Brilliant Pebbles'. Wood's long, gas-filled plastic tubes (quickly dubbed 'Brilliant Condoms') have come in for criticism, but Vice-President Dan Quayle was said to have been impressed. Earlier this year he directed NASA to search for more innovative concepts for space travel (see *Nature* 343, 301; 25 January 1990).

NASA's 'outreach programme' may turn up good ideas that would otherwise fall between the cracks, or never be raised at all, according to John Pike, a space analyst at the Federation of American Scientists. "They may not have the depth of analysis or the breadth of proponency" of an idea generated at NASA, he says, "but there may be something out there with some technical credibility that will allow NASA to do it cheaper." The problem is that any idea from outside NASA is unlikely to include a healthy role for all the NASA laboratories, something that Pike says has become an unstated prerequisite for the space agency's internal proposals. Pike intends to do his part and submit a proposal for a nuclear-rocket scheme that he first developed as a high-school science project and which, he claims, still makes sense.

Imaginative contributors should probably forget any thoughts of profit or even much fame. NASA says it wants "concepts", not specific products. Proprietary technology or anything patented is unwelcome for now. "We're trying to look at the big picture", said NASA exploration head Arnold Aldrich. The American Institute of Aeronautics and Astronautics is also to survey the aerospace industry for new ideas, and NASA will review research at the federal laboratories. "We want to make sure we're plugged in to what's available", said Truly at a press conference.

G. Christopher Anderson

Marshall Institute cuts no ice in Britain

London

MEMBERS of the George C. Marshall Institute, a privately funded US body which argues that the threat from global warming is overestimated, received a hostile reception from the British press and the Department of the Environment last week when they criticized predictions made by the scientific panel of the Intergovernmental Panel on Climate Change (IPCC). Professor William Nierenberg and Jim Frelk, directors of the institute, argue that current models do not predict climate change accurately enough to be used to set government policy.

The institute rose from obscurity last year with the publication of a report which suggested that global warming correlates more closely with solar activity than with the increasing concentration of greenhouse gases, and that moderate global warming over the next century could prove beneficial by countering the effects of an expected 'little ice age'. The Marshall report was well received in the White House, but the UK Department of the Environment regretted that the Institute of Energy, which invited Frelk and Nierenberg to London, chose to present their views at a press conference, rather than at a debate with the institute's many scientific critics.

Both Frelk and Nierenberg said that cuts in greenhouse gas emissions should be delayed until further research produces models that can make accurate prediction on the regional effects of climate change. Frelk, a political scientist by training, expects these models to be available in three to five years. But Nierenberg, director emeritus of the Scripps Institution of Oceanography, said after the conference that this was "too optimistic" and that ten years would be a more realistic figure.

Nierenberg said that estimates of future greenhouse gas emissions used by the scientific panel of IPCC (group I) in its deliberations are too large. IPCC's group I predicted that if no action is taken to curb greenhouse gas emissions, global mean temperatures will rise by 3 °C before the end of the next century. Nierenberg said that if the estimates are made by projecting recent trends in greenhouse emissions, rather than by making assumptions about energy use in developing countries, the predicted rise is only 1 °C. But Professor Tom Wigley, from the University of East Anglia, says that he has calculated future emissions based on trends over the past ten years which are "very similar" to figures used by IPCC. Wigley is an author of IPCC group I's report, and also briefed the Marshall Institute on its report. He says that the institute was very selective in its use of his data.

Peter Aldhous

Regulations breached

London

MEDICAL researchers at St Bartholomew's Hospital, London, transferring genes from the human gut parasite *Giardia lamblia* to *Escherichia coli*, broke UK regulations on the contained use of genetically manipulated organisms, according to Health and Safety Executive (HSE) inspectors who visited the hospital last month. It is the first recorded breach of the regulations.

Professor John Beringer, from the University of Bristol, who will chair a new committee to advise ministers on the release of genetically manipulated organisms into the environment, hopes the incident will draw attention to recent tightening of UK legislation. Genetic manipulation has become such a "simple" procedure, Beringer says, that some scientists are not aware of the regulatory framework that now exists.

Tony Taylor, secretary to the HSE's

I. M. Klotz and J. J. Katz

An apology

Nature wishes to put on record the following circumstances concerning the refusal of an article by Professor I. M. Klotz of Northwestern University and Dr J. J. Katz of the Argonne National Laboratory, both in Illinois.

Early in May, Klotz and Katz submitted for publication an article drawing attention to the close parallel between reports of the supposed discovery of *Acarus crossii* in the nineteenth century and those of 'cold fusion' that have more recently appeared. It was decided that the article should not be published in its original form, chiefly because of the required unhandy format (parallel columns detailing corresponding items of information). Two alternative courses of action were identified — either Klotz and Katz could be invited to recast their article or the author of the research that has uncovered the details of the *Acarus crossii* discovery, Dr James Secord, might be invited to write a separate article dealing with *Acarus crossii* alone. At no stage was it intended that the second course should be followed without consultation with Klotz and Katz.

In the event, through a series of errors in the *Nature* office, an article by Secord was commissioned, set in type and published without Klotz and Katz being consulted. *Nature* offers this apology to both authors for the distress and fury they have been occasioned as well as to Dr James Secord (who, needless to say, was throughout ignorant of the circumstances) for whatever embarrassment this regrettable affair may cause him.

John Maddox
Editor, *Nature*

Advisory Committee on Genetic Manipulation says that the researchers failed to notify HSE inspectors of their work, a legal requirement since 1978. St Bartholomew's lacked a Biological Safety Committee to oversee the work, and there had been no assessment of the health risk posed by the experiments. These two requirements were introduced last year.

Taylor believes breaches are "not a common occurrence" but, as with all statute law, "compliance is never 100 per cent". Julian Kinderlerer, from the University of Sheffield, a member of Beringer's new committee, is worried that some academic scientists regard the risks posed by their work as trivial, and are not complying with the law, but agrees with Beringer that ignorance of legislation may be a bigger problem. Until last year, the HSE had only to be informed about the initial manufacture of genetically manipulated organisms, but now their use must also be notified. "We have to make scientists aware" of their legal responsibilities, Kinderlerer says. Compliance in large industrial laboratories is thought to be better than in academic institutions but smaller companies may be more lax.

HSE has issued 'improvement notices', to Michael Farthing, of the gastroenterology laboratory at St Bartholomew's, and the City and Hackney Health Authority, which runs the hospital.

Experiments can continue, provided legal requirements are met in future. Taylor says that the HSE decided against harsher action — serving 'prohibition notices' immediately curtailing research, or prosecution — because the health risks posed were slight.

The breach at St Bartholomew's comes at a sensitive time as UK regulations governing environmental releases of genetically manipulated organisms are to be drawn up within the next 12 months, to comply with a recent European Communities directive (see *Nature* 344, 371; 29 March 1990). Beringer says that an important task for the new advisory committee on environmental releases, which meets for the first time in July, will be to help draw up these regulations. He acknowledges that public access to information on applications for consents to release remains a "contentious" issue.

The Environmental Protection Bill (see *Nature* 343, 4; 1990), currently passing through parliament, notes that some information about proposed releases may be withheld on the grounds of "commercial confidentiality". Beringer says that some researchers are also worried that publication of full details of proposed releases may lead to experiments being sabotaged by environmentalist activists.

Peter Aldhous

Who pays the bill?

Munich

WEST German Chancellor Helmut Kohl last week put strong pressure on the *Länder* to support an expanded science foundation for all of Germany. Speaking at the annual meeting of the West German science foundation DFG (Deutsche Forschungsgemeinschaft) in Bonn, Kohl said he supported the goal of a single science foundation for all of Germany, implying that he would back DFG's request for a large budget increase to underwrite East German research. The federal government and the *Länder* between them provide funds for DFG on a roughly equal basis.

DFG has asked for a series of increases, beginning with 9 per cent in 1991 and rising to 25 per cent in 1996, in order to handle the expected flood of grant applications from East Germany. Kohl, who appeared at the DFG meeting for the first time since 1986, did not commit himself to paying the full amount, but his speech was seen as a strong basis for negotiations in which the Bonn government will put pressure on the reluctant *Länder* to pay for at least part of the increase.

Spokeswoman Eva-Maria Streier pointed out that the increases would still be less expensive than setting up a second DFG in East Germany. The 1990 DFG budget is DM1,167 million (about \$700 million).

Streier explained that the extension of DFG to East Germany can proceed only if the East German government approves the move and the yet-to-be established *Länder* in East Germany pay at least a nominal contribution. When he visited DFG in May, East German Education Minister Hans-Joachim Meyer said that DFG grants are just what East Germany needs to improve its research, reported Streier. But there has been no official response as yet from the East German government as a whole.

DFG arrived at the 25 per cent figure by comparing the number of professors at universities in each country and assuming that East Germans would receive as many grants as West Germans do. Outside funds are sorely needed for research, especially in the academy, as previous sources, such as the soon-to-be-bankrupt state-owned companies called *Kombinate*, are drying up.

DFG has problems just supporting West German research. After years of increases that barely kept pace with inflation, DFG recently received a 5 per cent increase for 1990 and a promise from Bonn and the *Länder* that it would receive 5 per cent a year for five years, thanks to the intervention of Education Minister Jürgen Möllemann.

In a related development, Möllemann's

massive efforts on behalf of young researchers in West Germany were rewarded on 13 June when the Bonn cabinet agreed to invest at least DM4,000 million (about \$2,370 million) over ten years. If it is ultimately approved by the *Länder*, the new programme would mean an additional 12 per cent increase, apart from the 5 per cent already promised and the potential 9 per cent for East Germany, in 1991. The programme will increase stipends for both graduate students and young researchers seeking the teaching qualifications known as *Habilitation* with a special emphasis on supporting women in science. It is intended to help keep qualified young people at universities until professorships become available later in this decade.

Now that Bonn has been persuaded, DFG's chances of gaining extra funding for East Germany will turn on the attitude of the *Länder*. Both sides have to agree before a budget increase can take effect.

Hans Stender, spokesman for North Rhine-Westphalia, the most populous *Land* in West Germany, said his government supports the extension of DFG grants to East Germans. But he emphasized that the *Länder* in East Germany, which have yet to be formed, would have to pay their share out of tax revenues.

MAGLEV TRAIN

Japan bites the bullet on test track

Otsuki

DESPITE the gigantic cost, Japan's Ministry of Transport has given the official go-ahead for a project to build a test track for a high-speed magnetically levitated train through a mountain range west of Tokyo. The mountain track is essential to test the Maglev's capability to cope at speeds of 500 km per hour with the gradients, curves and tunnels that a proposed line between Tokyo and Osaka would encounter. If the line is built, it will carry passengers to Osaka in one hour, considerably less than half the time taken by the famous 'bullet' train.

The experimental track will run for 43 km between the tiny mountain villages of Sakaigawa and Akiyama near the town of Otsuki, about 90 km from Tokyo, and will cost \$2,000 million (¥304,000 million). It is expected to be completed in 1995 and will be underground for 80 per cent of its length. Until now, tests of the Maglev have been confined to a straight 7-km track on flat land in the southern island of Kyushu.

Otsuki was a natural choice because it lies on the proposed route to Osaka and its test track, which will be extremely expensive to build, could provide a foundation for the actual line. It is also no coincidence

Stender admitted that this seems unlikely, because the East German state is bankrupt and the new *Länder* will not be able to raise much in taxes until the economy gets on its feet. In that case, he said, Bonn will have to make up the difference. North Rhine-Westphalia considers all such special expenses generated by the absorption of East Germany to be "consequences of the war", and he pointed to a clause in article 120 of the West German constitution that declares that such costs must be paid by the federal government and not the *Länder*.

North Rhine-Westphalia has been the most extreme of the *Länder* in its criticism of the way science is supported in West Germany. Its Education Minister Anke Brunn made the revolutionary suggestion in February that the *Länder* should reduce their contributions to DFG from 50 per cent to 25 or even 10 per cent so that the money could be put to work closer to home.

No other *Land* took up her cause, and a Bonn Education Ministry official said that the suggestion would "bring on the ruin of the joint research funding system" that has worked so well in West Germany. A DFG official said that this plan would destroy DFG's autonomy by placing it under the thumb of the Bonn Finance Minister, who will not necessarily support the cause of science.

Steven Dickman

that Otsuki is in the constituency of Shin Kanemaru, an elder statesman of the ruling Liberal Democratic Party (LDP) who is a strong supporter of the proposed Maglev line.

Kanemaru and others are pitted against the Ministry of Finance which considers the Tokyo-Osaka Maglev line far too expensive to build — estimates range from ¥3 million million to ¥10 million million (\$20,000 million to \$67,000 million). There are still many technical hurdles to overcome before commercial operations can start, not the least of which will be finding a way to shield passengers from the strong magnetic fields of the train's superconducting magnets.

Such matters have not stopped land speculators from driving up prices in the Otsuki area. Land prices near the test track have soared to over ¥300,000 (\$2,000) per tsubo (3.3 square metres), ten times the price of five years ago. Estate agents from Tokyo have descended on the remote mountain villages near Otsuki offering wads of cash in exchange for land, but the local farmers are hanging onto their property in the belief prices will go even higher.

David Swinbanks

End of the beginning

Washington

ACTION to reform the state of US science education is finally getting under way after two years of complaints of falling standards. Last week saw two separate congressional hearings on bills designed to promote mathematics and science education and a national summit on the reform of science education organized by the Triangle Coalition — a group uniting 19 science and engineering societies, industry and 32 education associations. These developments came on top of the restructuring, announced earlier in the month, of the National Science Foundation (NSF)'s Science and Engineering Education Directorate and the surprise firing of its director by NSF chief Erich Bloch.

Reports showing that US schoolchildren come close to the bottom in international comparisons of mathematics and scientific achievement have appeared with almost monotonous regularity over the past few years. New impetus for change came when President George Bush set ambitious educational goals in his State of the Union address in January. By the year 2000, Bush demanded that US students reach a set of goals including being "first in the world in mathematics and science achievement". Although Bush did not spell out how to reach these goals, support for them came at a meeting in February of the state governors who carry much of the responsibility for US education.

Attempts to reform science education are thus "at the end of the beginning", according to the Triangle Coalition's executive director John Fowler. The coalition, which includes the American Association for the Advancement of Science (AAAS), the Federation of American Societies for Experimental Biology (FASEB) and the American Physical Society among its members, has attempted to define the opportunities for change in a series of reports over the past two years. Last week, it moved to the next stage with a triple offensive intended to build support for the rewriting of the school science curriculum, increase public awareness of the need for improved science education and expand local initiatives to help individual schools improve their standards.

Bringing in change is difficult because the 50 states of the United States (divided into 16,000 school districts) all have differing economic and social problems, standards of education and curriculum requirements. Reform movements are aimed both at the top-down approach of improving state science curricula (for which the Triangle Coalition appeals for support from scientific societies) and improving teacher training to the bottom-up approach intended to find local support

from industry to provide computers for schools, improve science museums and expand contracts between teachers and scientists in industry.

The National Science Foundation had been involved in the support of science education for years. But it was dissatisfaction over NSF's performance and the continuing debate over whether NSF puts its research budget before its education budget that led to a series of contradictory moves there over the past few weeks. Out went Bassam Shakhshiri, fired after six years as head of the Directorate for Science and Engineering Education, apparently because his demands for a bigger budget were too vociferous and sometimes appeared to by-pass Bloch. But in came a new, strengthened Directorate for Education and Human Resources, which will add programmes to encourage women, minorities and people with disabilities to study science to the programmes that had been run by Shakhshiri.

The increased stress on women and minorities comes because of a projected shortfall of scientists and engineers. Most US scientists and engineers are white males. Hispanics comprise 9 per cent of the population, but only 2 per cent of all

employed scientists and engineers. NSF predicts a shortfall of scientists and engineers of over 600,000 by the year 2020, owing to a declining interest in scientific careers and falling birth rates.

In Congress last week, the Senate Committee on Governmental Affairs held a hearing on a bill to establish a federal council to promote and coordinate mathematics and science education. Senator John Glenn (Democrat, Ohio) claimed that "lack of coordination" and inefficiency is evident in the thousand million dollars spent each year by more than a dozen agencies on science and mathematics education. In asking for increased federal control, the hearing represented one side of efforts to improve science education. The other, stressed at a second hearing at the House of Representatives subcommittee on science research and technology, included local efforts to interest people in science through better science teaching, including the use of interactive computer methods, and more attractive displays at science museums.

The Museum of Science in Boston already claims "more paid admissions" than to the city's baseball, football, hockey and basketball games combined. A newer success comes from the "Body-Tech" exhibition on which NSF has spent \$288,000, at the North Carolina Museum of Life and Science.

Shigeko Segawa

Green scheme avoids quarrels

Washington

THE four major US government agencies involved in mapping and sequencing of plant genomes agreed last week to join forces and create a single project, focusing on *Arabidopsis thaliana*, in order to avoid the kind of inter-agency rivalries that marred the beginnings of the Human Genome Project. The decision follows a meeting in Vienna earlier this month at which researchers from Europe, the United States, Japan and Australia agreed to coordinate national research efforts on the genome which are expected to cost \$100 million over the next ten years.

The National Science Foundation will lead US research efforts, coordinating the work of the National Institutes of Health, the Department of Energy and the Department of Agriculture. Combined, the four US agencies currently spend about \$6 million a year on *Arabidopsis*. But driven by support in the community for a large-scale project, they have asked for an additional \$20 million next year. In the United Kingdom, a new initiative by the Agricultural and Food Research Council will spend \$2 million on *Arabidopsis* this year. The European Communities, as part of their BRIDGE programme, plan to spend \$3 million ECU in 1991.

US plant genome researchers have taken pains to see that the effort concentrates on existing laboratories rather than new centres, a distinction that proved a problem for the Human Genome Project. Work is likely to be divided among existing laboratories. Two new biological libraries will store and distribute seeds and DNA fragments, and help distribute information and coordinate the project. Because the plant genome community is relatively small and closely knit, redundant research is unlikely to be a problem, says Chris Somerville, a Michigan state researcher who is a member of the project's steering committee.

The small size of the genome (at 100,000 kilobase pairs, it is about one thirtieth the size of the human genome), and the speed at which the plant reproduces, make it an ideal subject for study. Within two years, the project is expected to have a set of overlapping yeast artificial chromosome (YAC) clones which cover the entire genome. The work has been mostly done for about half of the project, says Somerville. Five years from the start of the project, all of the more interesting *Arabidopsis* genes should have been mapped and their functions explored. Five years beyond that, the entire genome should have been sequenced.

G. Christopher Anderson

NATURAL HISTORY MUSEUM

A tale of two seminars

London

SENIOR academics from across the United Kingdom were left dissatisfied after a meeting last Friday at the Natural History Museum in London that had been called to discuss the museum's future as a scientific research centre. The scientists had been invited by the museum's director, Neil Chalmers, so that he could answer criticism of his 1990-95 corporate plan, but after the meeting ended many complained that they had received no new information. The plan makes provision for far-reaching changes in the way research is organized at the museum, which will include the loss of one in six research posts.

The cuts are planned because nearly all the museum's government grant — more than £16 million — is consumed by salaries. "However much it hurts, we must lose posts", Chalmers reiterated at the meeting. In the plan, Chalmers has asked the Office of Arts and Libraries (OAL), the museum's main source of funds, to make up a shortfall estimated at £4.4 million over the next five years "so we can plan sensibly — we need that extra money just to stand still. We do have a real problem even if we do get this £4.4 million. If we don't, we face a devastating problem." Associate director John Peake presented the first stage of the museum's science strategy, which as yet goes little further than the broad statements outlined in the corporate plan.

The official presentation was preceded by a branch meeting of the Institution of

COMPUTER NETWORKS

NSFNET hits its stride

Washington

MAJOR portions of NSFNET, the massive scientific computing network that links more than 1,500 universities, research centres and supercomputer facilities, are to begin operation at 28 times their previous speed by the end of the year, the National Science Foundation (NSF) announced last week. With the upgrade, NSFNET will become the world's fastest computer network.

The \$7.9 million effort will raise the data-transfer rate for eight major US research centres from 'T1' to 'T3' speed — an increase from 1.5 to 45 million bits per second. Two new T3 nodes, which connect NSFNET to mid-level regional networks, will be added at the Massachusetts Institute of Technology and the Argonne National Laboratory. NSFNET established a T1-level connection to the European Centre for Particle Physics (CERN) several months ago (see *Nature* 344, 279; 22 March 1990).

G. Christopher Anderson

Professionals, Managers and Specialists (IPMS), the scientists' trades union. IPMS representatives called on the management to treat staff and their research with respect by abandoning the corporate plan, and giving substance to the advertised desire of the Prime Minister, Mrs Margaret Thatcher, to establish a Europe-wide systematic data-base in which the NHM could use its greatest strength — taxonomic research — to the full. This concern was voiced in the House of Commons by Mr Tam Dalyell, Labour MP for Linlithgow, Scotland. "It is a feature of the plan that the cuts do not relate to stated aims, even when the aims are the Prime Minister's", he said (*Hansard*, 11 June 1990; p.80).

At the official seminar, Chalmers said that NHM had taken the lead in coordinating museum research across Europe to avoid unnecessary duplication of effort.

The union meeting, also held in the museum, went ahead as planned despite, the IPMS alleges, efforts by the management to postpone it. Palaeontologist Dr Richard Jefferies, secretary of the IPMS science defence committee convened to draw attention to the cuts, addressed the meeting despite earlier threats of disciplinary action were he to do so. Jefferies likened the progressive attrition of funds at the NHM to the shrinkage of planarian flatworms during starvation: organ systems are lost in sequence, but the last to go is the nervous system. "That's the management", he said.

In what the IPMS criticize as an increasingly secretive management style, the provisions of 1990 corporate plan — unlike every other corporate plan since 1986 — were not discussed with staff before its completion. The trustees delayed approval of the plan from February until April, but its contents were effectively secret until 23 April, when staff were presented with what they see as a *fait accompli*.

Chalmers also noted the success of a new development fund (see *Nature* 241, 477; 1989) in raising money to put on new permanent exhibitions. These new displays will revamp a museum in which more than half the exhibition space is empty or has not changed for more than 15 years. But at the IPMS meeting, designer Ann Hollifield pointed out that to do this, the museum plans to bring in outside design contractors at the expense of the in-house team. Good exhibitions stem from a "marriage" between design and science, claims Hollifield, who is to be made redundant this autumn. The policy rethink is "throwing away years of investment in the in-house design team" and replacing the "marriage" with "a series of one-night stands".

Henry Gee

EUROPEAN COOPERATION

Environmental aid "a priority"

London

RESPONDING to frank descriptions from Eastern European environment ministers of the pollution problems they face, European Communities (EC) environment ministers agreed in Dublin last week that environmental protection should be given priority in the EC aid package for Eastern Europe. Non-EC European countries, including the Soviet Union, will also be free to join the EC's planned European Environment Agency — an environmental data-collection and monitoring service to be established later this year. The Dublin meeting was the first ever between EC and East European ministers.

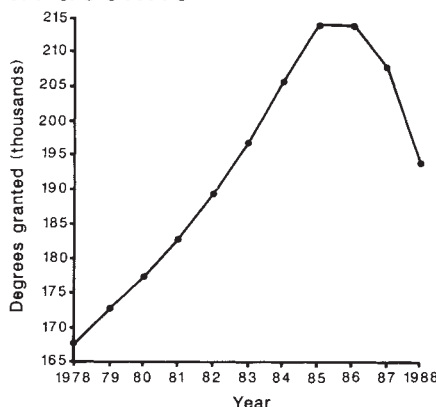
So far, only Poland and Hungary have received EC aid, gaining between them 300 million ECU (1 ECU = £0.70) with 49 million ECU earmarked for environmental protection. The Dublin agreement should mean that pollution control schemes will be emphasized in the additional 200 million ECU package planned for Bulgaria, Czechoslovakia, East Germany, Romania and Yugoslavia.

Polish environment minister Bronislaw Kaminski said that economic reforms are central to the fight against pollution. His government hopes that the removal of government subsidies on coal will reduce the country's profligate energy use.

Peter Aldhous

EDUCATION

United States science in decline



THE number of US students receiving undergraduate degrees in the natural sciences dropped nearly 10 per cent over two years, according to National Science Foundation statistics. Defined as the fields of engineering, physics, chemistry, mathematics, computer science, biology and environmental sciences, the group suffered a loss of more than 20,000 degrees between the years 1986 and 1988. During that period, the overall number of degrees granted to US students in all fields rose by nearly 6,000, to an all-time high of 1,006,033.

G. Christopher Anderson

Natural History Museum (cont'd)

SIR—In a recent News article (*Nature* 345, 4; 1990), it is implied that the new plan at the British Natural History Museum (NHM) to abolish tenure and emphasize short-term appointments finds a model in practices at the American Museum of Natural History. This is inaccurate. Here we hold to a programme of permanent appointment for curators, scientific assistants and technicians. This programme is complemented by support for graduate and postgraduate fellows. The fellowship programme — which hardly fits one's idea of 'short-term contract work' — was anchored from new sources of funding. No curators were dismissed as redundant in order to provide for fellowships. In lacing together the activities of permanent staff with that of postdocs and graduates, we are like the major research-orientated universities of the world. In fact, we have formal agreements for graduate training with Columbia University, Cornell University and City University of New York. These important institutions recognize that we can provide a dimension of science training that even the strongest university programmes cannot duplicate.

Museums should, of course, strive to provide leadership in science, to open outward to the public, and to forge educational links with universities. New directions should, however, build on strengths of programmes already established. It is a bitter irony that at the very time when

there is a renaissance in the field of systematics, and growing concern for the loss of biodiversity, we are confronted with funding constraints and proposals for the NHM that threaten one of the great centres for systematic research.

The NHM is one of a few institutions that can claim to provide a formidable sampling of the vast richness of life on this planet. Its collections and its curators belong to the world, not just to London or the British Isles. Centres with appellations such as 'biodiversity' and 'living resources' — programmes now planned for the NHM — are sprouting up all over the place. There is, however, no other bird collection like that at the British Natural History Museum. As an educational and public institution, a museum is most credible if it maintains vital research programmes that are actually relevant to the priceless repository that it protects and displays.

MICHAEL NOVACEK

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SIR—What I read of the fate of the British Natural History Museum is astonishing. How can we imagine the demise of most activities in systematics in an institution — the reference institution — devoted to natural history? How can it be possible to think of a proper curating of collections

ness of German administration. . .". What Heisenberg was actually doing was lecturing on physics, getting confiscated equipment returned to physicists in occupied countries, and aiding his colleagues in other ways.

CONSTANCE A. WARNER

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How synthetic?

SIR—The notion of 'synthetics' in the context of the economic growth/no-growth debate can at times be misleading.

You write, for instance (*Nature* 344, 179; 1990), that "Some natural resources have gone for good, but have been used in part to generate the skill with which they can be replaced, naturally or by synthetics".

Nowadays, the most widely used synthetics are derived from petroleum, which is itself in shorter supply on this planet than the iron which the synthesis usually replace.

Ceramics would be a better bet, if they can be made more versatile.

CARLO PISCICELLI-TAEGGI

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that constitute a world heritage without competent scientists? What is the rationale in separating curator activities and research activities? I read (*Nature* 345, 191; 1990) that the "basic problem is money". An uneducated attitude is to think that money for natural history is a waste of money. Who will believe that there is less money in the United Kingdom today than 20 years ago?

The director says (*Nature* 345, 198; 1990) that "the only way is for our museums to be selective". It is easy to see that this kind of selection may be the beginning of the end, particularly when this selection applies to "taxonomic research" in non-taxonomic areas such as "environmental quality, mineral resources [and] human health".

Behind the new magic word "biodiversity", as an example, I see the demise of the fossil mammal section, where excellent research is done. To suppress natural history research in the Natural History Museum is like a story by Lewis Carroll. A pure nonsense.

P. TASSY

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SIR—The news of the latest changes at the Natural History Museum (NHM) in London is sad and depressing for every biologist. The proposed changes are particularly distressing in an institution of such great international importance, and would undermine research in the entire field of systematic biology.

These changes, so deleterious for the future of comparative neo- and palaeobiology, are being introduced at a time when the 23rd General Assembly of the International Union of Biological Sciences (IUBS) has appealed to the international community of biologists to create conditions that will enhance the further growth of taxonomy. The union has promoted as one of its current research programmes studies on biodiversity in the context of ecosystem structure and stability. I would expect from responsible authorities a better understanding of the role of the NHM, serving the international biological community and shaping the progress of research on biological diversity on a global scale. I trust that solutions can be found to permit continuation of the present scope of research at the NHM, preserving its magnificent scientific heritage, both national and international.

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Heisenberg's principles

SIR—Mark Walker, in his book *German National Socialism and the Quest for Nuclear Power 1939–1945* (reviewed by John L. Heilbron in *Nature* 343, 421; 1990) draws strange and convoluted inferences from events cited out of context and from documents that are described but seldom quoted. Heisenberg's private conversation with the Dutch physicist Heindrik Casimir, for example, is interpreted as somehow extending to, and alienating, all Heisenberg's Dutch colleagues. Heisenberg's lectures on physics at German cultural institutes are construed as full support of the German war effort, as "helping Germany win the war . . .". It is interesting to note that it has recently been revealed that there was a spy in Heisenberg's circle, probably Karl-Friedrich Bonhoeffer, who reported to publisher's representative Paul Rosbaud in his *alter ego* as a British agent.

Heilbron, in his review, describes some German scientists as "tainted", and Heisenberg as "travelling up and down the occupied territories as an ambassador of German culture, justifying the ruthless-

Animal experiments

SIR—We write to protest strongly against the article by Peter Aldhous (*Nature* **345**, 190; 1990). We are all former colleagues of Professor Wilhelm Feldberg from soon after he succeeded Sir Lindor Brown as head of the division of physiology and pharmacology at the National Institute for Medical Research, formerly directed by Sir Henry Dale. We have known and admired the work Feldberg has carried out for nearly 70 years, over 50 of them in this country. We offer no comment about the allegations reported; we have not seen the 'evidence'. We would merely say that it is difficult to believe that a man so kind and gentle, so careful and meticulous over his experiments, could conceivably be guilty of deliberate cruelty to any animal or any human being.

We object first to the lack of any adverse comment on the behaviour of the two people who offered 'evidence' to the Advocates for Animals. It is indeed typical of Feldberg's kindness and consideration that he was ready to invite them into his laboratory. *Nature* does in fact refer to "deception" and "pretence"; but it is difficult to imagine a sorrier tale of lies, fraud and deceit than is to be found in the two years' effort of Miss MacDonald and the five months' effort of Mr Huskisson. As to any alleged contravention of the 1986 act, it is perhaps worthy of note that it took them a remarkably long time to obtain, or to report, any 'evidence'.

Our second objection is that there is no indication in the article of the very great value of the absolutely fundamental contributions made by Feldberg. These have been first in our understanding of allergy, and then in establishing chemical transmission at the adrenal gland, the neuromuscular junction and the ganglionic synapse, and more generally in developing the concept of neurohumoral transmission at synapses and by neurons of central origin. He is held in high regard and affection by the scientific world, recognized among other ways by his Royal Medal and his CBE.

The only mention in *Nature* of Feldberg's distinction is in relation to the possibility that it may have exempted him from proper monitoring. The first mention made of him refers to him simply as "an 89-year-old researcher". This is a sad lack of understanding of a lifetime of brilliant pioneering scientific work.

We would have expected *Nature* to have given a more balanced account of this most unfortunate incident, to have shown a very much more sympathetic attitude towards a great man of biomedical science, and to have condemned out of hand the disgusting methods used to col-

lect the 'evidence' against him, which disfigure the animal welfare movement.

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SIR—Two aspects of your articles (*Nature* **345**, 189–190; 1990) concerning the Feldberg case indicate that the alleged misconduct is not an isolated instance. The fact that the scientific community involved accepted the pretence that the videotapes of the scientific experiments were to be used to teach US students leads to the conclusion that the nature of these experiments was either not realized or was accepted as necessitated by scientific needs. The fact that Wilhelm Feldberg is a fellow of the Royal Society and was recently awarded a medal by the British Pharmacological Society shows that this type of animal experimentation is compatible with an honoured position in the scientific community, and may in fact form the basis on which a scientific career can be grounded. It is impossible to believe that all these aspects should have gone undetected during Feldberg's scientific career and in the National Institute for Medical Research.

What can be done to protect animals, science and researchers from such events? As well as supporting and developing all the control mechanisms described in your article, the following measure would be very valuable: European and North American governments should decide that scientific work involving animal experiments can be published only in selected scientific journals which will be granted a special licence for this purpose.

The licence should be given only to journals that fulfil two criteria: (1) the journal should have a wide international distribution and readership; (2) manuscripts published in the journal generally should have a high impact on the development of the scientific field in question (as measured for example by citation). In practice, only a small number of well-known and respected journals in each field would qualify for such a licence.

This restriction if properly enforced would: (i) slow down the flood of publications containing animal experiments that show almost nothing, except for the phenomenon observed, and which are

published in an increasing number of scientific journals; (ii) simplify analyses of the amount of scientific work involving animals; (iii) improve the quality and the dissemination of scientific work involving animal experiments.

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Drug regulation

SIR—In response to Burton Appelton (*Nature* **344**, 99; 1990), it would seem that when at the Food and Drug Administration (FDA) he was a man ahead of his time. The questions that he was raising 25 years ago concerning drug stereochemistry are now being raised with some frequency by the agency. Twenty-five years ago, the US regulatory framework was silent on the subject of drug stereochemistry. However, many parts of Section 505 of the Food, Drug and Cosmetic Act (including those cited in Appelton's letter) could be interpreted as addressing drug stereochemistry. But an FDA guideline published in 1987 (Guideline for Submitting Documentation in Drug Applications for the Manufacture of Drug Substances) does provide some general guidance. Its language has prompted much discussion between the pharmaceutical industry and the FDA. The statement made in a recent Review Article on asymmetric synthesis (*Nature* **342**, 631; 1989) which suggests that regulatory agencies are exerting pressure on pharmaceutical companies to develop enantiomerically pure drugs is true.

Discussion of the current FDA regulatory environment concerning drug stereochemistry can be found in a recent article by DeCamp (*Chirality* **1**, 2; 1989). A description of current FDA activities aimed at promulgating regulatory guidance in this area can be found in an article by Weissinger (*Drug Information Journal* **23**, 663; 1989). The Pharmaceutical Manufacturers' Association's position on this subject will be found in a recently published manuscript (*Pharmaceutical Technology* **14**, 46; 1990). A summary of worldwide regulatory environments and current trends within the pharmaceutical industry regarding issues of drug stereochemistry will be found in a manuscript I have written (*Annual Reports in Medicinal Chemistry* **25**, 323; 1990).

Today, matters of drug stereochemistry are becoming among the most complex and important issues in pharmaceutical research and development.

MICHAEL GROSS

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Trouble at Rome University

SIR—We would like to bring to the attention of the international scientific community the situation at the faculty of medicine of the University of Rome 'La Sapienza'.

This school of medicine, one of the largest in the world, has more than 10,000 students and a teaching staff of over 800, consisting of about 200 full professors and 600 associate professors, all of them with tenure. Unfortunately, the scientific output of this conspicuous institution is far from adequate for its size. The annual production of papers in international refereed journals averages 80; that is, 0.1 per faculty member. At a time of vigorous growth of new and promising directions of biomedical research, such a mediocre level of scholarly activity is startling, and this situation reflects a lack of involvement in research for most of the faculty members, perhaps as a consequence of their high mean age (over 50 for associate professors).

This situation might have been partially remedied by the appointment of younger staff, better trained and more keen on research. Indeed, every few years (generally four) the government asks the faculties to plan their development in terms of new teaching positions. In this context, a four-year plan including proposals for new positions of associate professor was to have been presented to and voted on by the majority of the faculty members no later than March 1989. But the faculty of medicine in Rome has succeeded in postponing any decision for over a year, and has recently decided, uniquely among all the Italian faculties, to renounce the appointment of new associate professors, including those proposed from a list of candidates who have spent at least three years at distinguished foreign research institutions. This negative decision was taken in opposition to the recommendations forwarded by the departments and institutes.

We feel that blocking the appointment of new staff members at associate professor level will result in strangling the development of new research areas and worsening the scientific and teaching stature of this school of medicine at a time when the international scientific community is vigorously expanding in new research directions in the field of biomedicine.

This destructive opposition was promoted by a group of associate professors who hold as hostage the appointment of new associate professors, regardless of teaching necessities and research development, until the global number of full professors approaches that of associate professors. The latter result is to be obtained by a sort of qualification test which would promote

the present associate professors to full professors, and automatically confirm their tenure at Rome.

This self-interested behaviour testifies to a short-sighted view of the future of medical and basic science, and will soon produce a sclerotic institution no longer able to cope either with teaching needs or with the quality standards of the scientific community. This attitude sacrifices the scientific growth of the faculty to the career expectations of its members and is in line with the archaic faculty tradition which has in the past resulted in favouring an internal member of the faculty over the Nobel laureate Dr Bovet.

We believe that something should be done to change this anticultural and narrow-minded approach. Denouncing this situation is a duty to the scientific community at large and even more to the young and qualified researchers whose prospects of contributing to biomedical research and teaching in Rome are being frustrated.

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Testing criteria

SIR—The implication in your leading article (*Nature* 343, 494; 1990) regarding the toxicity and efficacy tests for genetically engineered 'drug' products, that such products need not be subjected to the rigorous criteria applied to chemically synthesized drugs because "they are intended to be identical with those produced naturally in normal human beings", should not be allowed to pass unchallenged.

The intention and the outcome of any process are frequently very different and a number of problems can be envisaged in the case of genetically engineered substances.

Besides contamination from constituents of the culture, used in their production, having properties that preclude their separation from the product, a far more serious problem could arise from errors during the synthesis and replication and/or the misreading of the recombinant DNA. The products of such events — a single amino-acid substitution due to a single base change in the DNA or an alteration in a peptide sequence due to a frame-shift type mutation — could result

in serious side effects such as an alteration in the nature of the activity, for example antagonist rather than agonist activity and/or, the acquisition of immunogenicity by the altered product that would cross-react with the "products produced naturally in normal human beings" leading to an autoimmune reaction.

Rather than being "over-rigorous", it would be more prudent to ask whether the tests applied are the correct tests for such products and are sufficiently rigorous.

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Big guns

SIR—That which has been thought about has been, is being or will be tried out, particularly on the wilder shores of defence science. Both your comments on 'big gun' technology and the free-ranging thoughts of Daedalus (*Nature* 344, 811 & 820; 1990) have been witness to this.

Some 30 years ago when studying the storage of high-pressure air in chalk caverns, I was discreetly approached to comment on the feasibility of a deep shaft to house a giant multi-stage air gun — a British rival to HARP. A decade later, I used a designed sequence of explosive impulses to liquefy sand. Such impulses designed along Daedalus lines have been used to test full-scale structures for their response to earthquakes. True novelty is rare.

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Weight of culture

SIR—*Nature* suffers from tunnel vision in smugly condemning Americans for preferring the British system of weights and measures (*Nature* 344, 575; 1990). This attitude fails to appreciate that units of measurement are not merely calculating devices, but integral components of a nation's cultural matrix. As such they are the numerical equivalents of the languages, traditions and customs that identify and enrich us both collectively and as individuals.

The ineluctable march of the metric system represents the victory of cold calculation over the ebullience of the human spirit. Once its triumph is complete, the world's cultural gene pool will have become further depleted and humanity reduced one more step towards the mentality of the average robot.

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Duesberg, HIV and AIDS

Robin A. Weiss and Harold W. Jaffe

Last century there was a sharp difference of opinion between those, such as Koch and Pasteur, who proposed that disease could be caused by invisible microbes, and others who held that epidemics are the result of evil vapours (mal'aria). Arguments that AIDS does not have an infectious basis are as quaint as those of the miasmalists.

A TRAGEDY on the scale, novelty and social dimensions of AIDS inevitably attracts explanations that betray the obsessions of their purveyors while bearing little relation to the epidemic. Some of the oddest ideas have come from scientists and physicians. We have, for example, been told that the human immunodeficiency virus (HIV) originates from outer space, or as a genetically engineered virus for germ warfare which was tested in prisoners and spread from them. Peter H. Duesberg's proposition^{1,2} that HIV is not the cause of AIDS at all, is, to our minds, equally absurd.

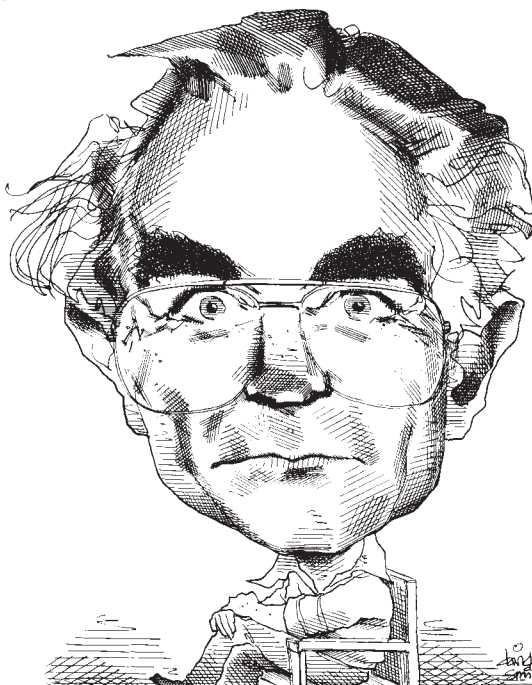
Even though the scientific community felt that Duesberg's argument had been amply refuted³⁻⁵, his ideas have gained a sympathetic hearing in the media. Last week a British television programme, "The AIDS Catch", provided Duesberg with a new forum; the programme has been widely acclaimed, even though it did not attempt to state the evidence for the mainstream, scientific view that it sought to demolish. As Walter Gilbert pointed out, challenging dogma is a vital process in scientific discovery, and Duesberg has indeed forced many of us to question more deeply the role of HIV in AIDS. It is in this popperian spirit that we think Duesberg's theory is proven false and needs amendment.

Duesberg's special theory is that HIV cannot be the cause of AIDS². His general hypothesis, extolled in the television programme, is that AIDS is not infectious at all. Duesberg and Jad Adams, the journalist who has championed his ideas⁶, dismiss arguments that AIDS has developed in people accidentally inoculated with blood containing HIV as insufficient to stigmatize HIV, as the virus was not first isolated in pure form and the blood may have contained other immunosuppressive factors. In particular, Duesberg argues that HIV does not fulfil Koch's postulates.

Robert Koch drew on his mentor Jakob Henle's ideas and his own experiments on the culture and re-inoculation of the anthrax bacillus to formulate his postulates⁷ for identifying the causes of infectious diseases. They may be summarized as follows: (1) The agent occurs in each case of disease and in amounts sufficient to

cause pathological effects. (2) The agent is not found in other diseases or as a non-pathogenic parasite. (3) After isolation and serial propagation in pure culture, the agent can induce the disease anew.

Duesberg argues that HIV fails each of the three postulates — first, that too few



Peter Duesberg — a perilous message.

T lymphocytes in the peripheral blood are infected to cause immune deficiency; second, that asymptomatic HIV carriers exist; and third, that HIV isolated in pure form has not been shown to induce AIDS in humans or animals. He is wrong on the first count and correct on the other two, but what seems bizarre is that anyone should demand strict adherence to these unreconstructed postulates 100 years after their proposition. In fact, Koch discussed the difficulty of fulfilling the third postulate for tuberculosis and cholera when he first enunciated them⁷.

Koch later abandoned the second postulate altogether when he discovered asymptomatic carriers of cholera⁸ and later 'Typhoid Mary'. Similarly, all virologists appreciate that poliovirus causes paralysis in only a few infected subjects, yet the very success of polio vaccines in preventing the disease supports the conviction that the virus is the causative agent.

The discovery of viruses and the study

of chronic diseases led to valuable amendments and additions to Koch's postulates by T.M. Rivers, R.J. Huebner and others, which have been wisely and lucidly reviewed by Alfred S. Evans⁹. Evans has also examined the case for HIV as the cause of AIDS by these criteria, and pronounced the virus guilty⁵. It is ironic that, in the same month as the latest outburst challenging the role of HIV, a close relative, the simian immunodeficiency virus (SIV) should be shown to cause AIDS in monkeys after molecular cloning, thus satisfying Koch's third postulate. Two reports^{10,11} discussed by M. Martin in *News and Views*¹² last week by have shown that SIV rescued from recombinant DNA clones causes the slow and acute disease characteristic of the original SIV strains. Moreover, protection against simian AIDS is conferred by prior immunization with inactivated SIV¹³. These studies would surely have pleased Koch, the inventor of cloning. A century later we have serology and molecular probes to provide evidence of infection, which in the case of HIV and AIDS fits Koch's first postulate as well as does disease resulting from acute infection.

Duesberg declares that AIDS does not have an infectious aetiology because "viruses and bacteria work fast or never". Here he confuses viral replication at the cellular level with disease in the infected individual. By his logic, mycobacteria do not cause tuberculosis and leprosy, trepanemes do not cause tertiary syphilis, hepatitis B virus does not cause chronic liver disease or liver cancer, and the lentivirus visna/maedi in sheep does not cause the insidious slow wasting and neural degeneration seen months or years after viral infection. Persistent infections do cause disease, often after long latent periods and without acute effects¹⁴. Viruses infecting lymphocytes, macrophages and the brain, such as visna or lymphocytic choriomeningitis, are apt models.

Duesberg also asserts² that viruses do not cause disease after specific neutralizing antibodies appear in the blood; hence, HIV cannot cause AIDS. Most persistent infections, however, proceed in the presence of a humoral immune response¹⁴. In some diseases, such as Dengue fever,

antibodies to the virus may even exacerbate the disease; in others, such as chronic hepatitis, cellular immune responses to viral antigens may be an important component of the viral pathogenesis. So there is nothing peculiar about HIV and AIDS.

Another tenet of the case against HIV is that a virus found in only 1 in 400 peripheral blood lymphocytes at any one time cannot engender immunodeficiency. This, of course, belies the fact that blood, while being the easiest tissue to sample, is not the only one infected by HIV, so that the main reservoir of infected cells may be in the bone marrow, lymph nodes and tissue macrophages. It also ignores indirect mechanisms for T-cell depletion^{15,16}, and that cells killed by HIV will be rapidly removed from the circulation.

It is unwise to conclude that because we do not understand the pathogenesis of HIV in molecular detail, it is therefore harmless. We have yet to meet a pathologist who can describe exactly how the variola virus caused death, and precisely what determined who recovered and who died from smallpox. The minor changes in nucleotide sequence that determine whether poliovirus causes a potentially lethal, paralytic destruction of motor neurons rather than an asymptomatic gut infection have been mapped, yet we still do not understand the cellular mechanism of the tissue tropism. We know even less about why SIV seems to be harmless in mangabeys and lethal in macaques. So Duesberg is right to draw attention to our ignorance of *how* HIV causes disease, but he is wrong to claim that it does not.

The evidence that HIV causes AIDS is epidemiological and virological, not molecular. The infectious, transmissible nature of the underlying cause of AIDS has been apparent from early contact-tracing studies¹⁷ and since the first cases appeared in recipients of single blood transfusions, before the discovery of HIV (ref. 18). Because the virus is new to most of mankind, it fits the pattern of the AIDS epidemic so well one could not invent a better textbook example. In every country and city where AIDS has appeared, HIV infection preceded it by just a few years, from its early spread in central Africa and the United States, through Rio de Janeiro and Bangkok to the appalling recent news from Romania and the southern Soviet Union. In every social group at risk of AIDS, HIV has preceded the disease. The temporal pattern and diverse risk groups are strong evidence against 'lifestyle' explanations. HIV is the singular common factor that is shared between AIDS cases in gay men in San Francisco, well nourished young women in Uganda¹⁹, haemophiliacs in Japan and children in Romanian orphanages.

The tight association of HIV and AIDS in risk groups is not the only causal evidence. Case-control studies show that

within risk groups it is only the HIV infected who develop AIDS (for example among haemophiliacs²⁰ and their wives, or in the infected infants compared to the uninfected siblings of HIV-positive mothers). Numerous cohort studies show, sadly, that the virus does not act "early or never", for each year following HIV infection brings a further crop of newly diagnosed AIDS cases^{21,22}. In the television programme, Duesberg complained that orthodox AIDS researchers continually "shift the goal posts" in defining AIDS. The list of clinical manifestations resulting from immune suppression and neurological disease certainly requires periodic revision as our knowledge grows. Because other causes of immune suppression also give rise to similar symptoms, there will be HIV-negative individuals who fall within the Centers for Disease Control definition of AIDS. The relative risk of AIDS among HIV-infected people, however, compared with HIV-negative people in each risk group, is much greater than the relative risk of lung cancer in smokers.

A case in point is the occurrence of HIV-negative Kaposi's sarcoma (KS)²³. In fact, 14 KS cases in HIV-negative American gay men have been reported against 12,764 cases in HIV-positive homosexuals or bisexuals²⁴ (HIV relative risk in KS 900:1). Kaposi's sarcoma is a lesion that also occurs in immunosuppressed organ transplant patients (relative risk 400:1). It is probably due to an unknown sexually transmitted agent²⁴, but with a 900-fold higher risk in homosexuals it is dangerous to dismiss the role of this virus.

In the face of compelling epidemiological data causally linking HIV with AIDS, one need not harp upon molecular quibbles, important though these are for directing research to the prevention or amelioration of HIV infection. To deny the role of HIV in AIDS is deceptive. One senses the desperation of the case when it shifts away from scientific argument to casting aspersions on the motives of HIV research in the 'AIDS industry'. Virologists and immunologists are supposed to be the pawns of a collectively benighted pharmaceutical industry or victims of sterile and blinkered orthodoxy⁶. We in turn regard the critics as 'flat-earthers' bogged down in molecular minutiae and miasmal theories of disease, while HIV continues to spread.

There is an interesting twist to the recent arguments of the HIV detractors which was illustrated in last week's television programme. Although most of the programme supported Duesberg's view, one sequence laid emphasis on infectious cofactors, in particular Luc Montagnier's current view that certain mycoplasma infections may exacerbate progression to AIDS in HIV-positive people. But two infections do not add up to none.

In any disease with a long and variable induction period, cofactors play a significant role, whether they be genetic, environmental, dietary or other infections. Last year it was claimed that cytomegalovirus is an important concurrent infection in AIDS progression²⁵; now it is the turn of mycoplasma and human T-cell leukaemia virus²⁶.

The confusion lies between what is necessary and what is sufficient to cause AIDS. With a mean time of 8–10 years for the disease to become manifest, one would expect a number of cofactors of HIV to exert a considerable effect. But if the primary cause were eliminated — in the case of AIDS by an efficacious HIV vaccine — then the disease should disappear in time. What is surprising is how few cofactors are apparent in AIDS so far, among them HLA (ref. 27) and age^{20,21}.

Peter Duesberg is said to be contemplating the emulation of Max von Pettenkofer (who drank a *Vibrio cholera* culture) by inoculating himself with cloned HIV. Surely you're joking, Mr Duesberg! One hundred and fifty years ago, John Snow's brilliant epidemiological investigations led him to remove the handle of the Broad Street pump, thereby stopping the cholera epidemic in Soho, London, and lending support to the germ theory of disease. If Duesberg's critique of AIDS were as rigorous as Snow's analysis of cholera, that would be a signal service. But if he and his supporters belittle 'safe sex', would have us abandon HIV screening of blood donations, and curtail research into anti-HIV drugs and vaccines, then their message is perilous. □

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New ways with aggregation

A new treatment of certain problems of aggregation, neat in itself, promises to be a helpful adjunct to established (but imprecise) methods.

SIMPLE-MINDED problems usually conceal a catch somewhere. Here is one, taken from *Physical Review Letters* for 11 June. Suppose there is a gun that fires bullets in the same direction at regular intervals. To simplify (and to make the problem one-dimensional), suppose there is no such thing as gravity. If all the bullets left the muzzle with the same velocity, there would be no problem, but merely a train of bullets following each other at regular intervals in the direction of the axis of the barrel. In that case, there is nothing much to say.

The fun begins when you suppose that the muzzle velocities are not all the same. Then, some bullets will overtake those ahead of them and may then stick together. Given enough variability of muzzle velocity, substantial aggregations of bullets, all travelling in the same direction, may be formed. G. F. Carnevale and W. R. Young, from the Scripps Institution of Oceanography in La Jolla, California, and Y. Pomeau, from the Ecole Normale Supérieure in Paris, call it "ballistic agglomeration" (*Phys. Rev. Lett.* **64**, 2913; 1990). The surprise is that nobody seems to have thought of tackling it before this. The authors' acknowledgement of support from the US Office of Naval Research should not be taken too literally; even in naval battles, shells do not often collide with each other, but vortices and convection plumes do so routinely.

What are the simple expectations of the problem? Both mass and momentum will be conserved at every collision. The velocities of individual projectiles can be taken to be randomly distributed in a finite range about some mean value. What can be said about the long-term behaviour of such a system? Some may say nothing. Carnevale *et al.* suggest that others, more sophisticated, and brought up on textbooks of fluid mechanics, will argue that the pattern of agglomeration a long way from the muzzle will depend on the mass flux (which is constant and equal to m_0/τ_0 , where m_0 is the mass of each projectile and τ_0 the interval between firings), the momentum flux (also constant and equal to $m_0 u/\tau_0$ where u is the mean velocity) and the distance from the muzzle (x).

Distance, here, is a proxy for time, and it would be reasonable to ask for some answer to the question of how the masses of the agglomerations reaching x are distributed; some will consist of single bullets, others of several. Textbook writers have a ready solution. Since there

are three variables (two fluxes and a distance), there is only one way of making a function which has dimensions of mass and which is itself a function of x .

This simple argument leads to the conclusion that the distribution of mass is the function $F_p x/F_m$, where F_p and F_m are the fluxes of momentum and of mass respectively. The immediate thing to say about this result is that it is incorrect. The authors use a numerical simulation to show that even when a train of 100,000 projectiles has been launched, with velocities taken randomly from an interval 50 per cent above and below the mean, there are fewer than 1,000 agglomerations in being, but that many of these consist of only a single projectile. The surprise is that there are so many singles left.

Evidently this is an over-simple version of what must be the real problem — that of telling what happens to aggregating particles which are free to move in several dimensions. The authors rightly say that aggregation by coalescence at every collision is a very special case of a more complicated phenomenon. The surprise, again, is that while some things can be said about the general problem, only the one-dimensional case has been dealt with. But the argument is memorably neat.

To visualize what happens at agglomeration, the authors assume that every aggregation of unit particles assumes the configuration of a sphere. The problem is to find the mass distribution as a function of time, t . Because of the adjustment to a sphere, $m(t) = r^D(t)$, where $r(t)$ is the radial distribution and D the dimensionality. At the very outset of the agglomeration, the interval between collisions will be the ratio of the volume of each particle to the volume swept out in unit time — specifically, $a^D/u_0 r_0^{D-1}$ where a stands for volume and the zeros emphasize that this is right at the beginning.

Now what happens as time goes on, say after the passage of, say, q units of the characteristic time τ_0 ? Evidently some unit spheres will have agglomerated into larger structures; indeed, the expected number of units in each agglomerate will be some function $N(q)$. But the problem is then the same as at the beginning except that the particles are larger. This already says some useful things — the velocity of the aggregations declines inversely as the square root of the function $N(q)$, for example.

Now comes the trick. The authors choose a second later time, and regard

that at $q\tau_0$ as the starting point, which leads to a similar set of scaling rules, which must be identical in form with the first. The end result is that $N(q) = q^{2D/(D+2)}$ (where D is dimensionality). In short, in one dimension, particles grow as $q^{2/3}$, in three dimensions as $q^{6/5}$. There are numerical solutions (in one dimension) to prove the point.

Two interesting issues arise, of which the most obvious is why so much hangs on simulation in one dimension. The explanation is humdrum — the algorithms are tractable only if the particles are very small.

The more interesting issue is what might be expected to come out of studies such as these. The authors list a whole series of problems to which their work may be relevant, ranging from the condensation of stellar nebulae into planets to the aggregation of colloids and the interaction of hydrodynamic structures. Although all these are problems which can be (and have been) tackled differently, a new way of working may prove especially valuable.

So much is nicely illustrated by the best-known model of aggregation, that of "diffusion-limited aggregation" originally due to Edward Witten at Santa Barbara, California. There the guiding idea is that particles diffuse towards a growing aggregate by random walks from a remote periphery, and stick whenever they reach a site (on a normally two-dimensional lattice) adjacent to another already occupied. Unvarnished, this process builds lacy structures whose dimensions prove to be fractional — they are fractal structures, yet many hours of elaborate central processor time spent on the numerical simulation of such models have not solved many practical problems — but there are some intriguing applications in the scattering of light by smoke particles. It is much more important that this model has lent itself to the prediction of some general scaling laws, and to a kind of intuitive sense of the physics of the problem.

Much the same will probably happen with its new model. Apart from its problem of planetary aggregation (which is essentially three-dimensional) there are few obvious applications likely to be of direct utility. Plainly, one should not expect more. Despite the effort and the time that goes into the development of these models, their lasting value is probably heuristic — people have a better insight than previously. **John Maddox**

A diversity of diabetes

Peter Parham

ECCENTRIC Japanese mice have greatly contributed to immunology. At the turn of the century, research on Japanese waltzing mice opened the door on tumour transplantation and discovery of the major histocompatibility complex. Using a more recent import, the non-obese diabetic mouse, three groups¹⁻³ whose reports appear on pages 722-729 of this issue now provide insight into human diabetes.

Non-obese diabetic (NOD) mice have an inherited predisposition to autoimmune disease resembling human insulin-dependent (type 1) diabetes mellitus⁴. Several genes are involved, a large contribution coming from the major histocompatibility complex (MHC). Amino acid sequence comparisons have revealed a provocative correlation between disease susceptibility and substitutions at position 57 of the β -chains of human HLA-DQ class II MHC molecules and their murine homologues, I-A. It has been claimed that aspartic acid at position 57 (Asp 57) provides resistance to diabetes, whereas other amino acids are associated with susceptibility⁵. Fitting this correlation, the single class II molecule of the NOD mouse (I-A^{NOD}) has serine at position 57 (ref. 6). Using transgene therapy, the three groups now find that diabetes is prevented in NOD mice receiving an additional Asp-57-containing class II molecule.

Murine diabetes

As in humans, murine diabetes is caused by autoimmune destruction of the pancreatic β -cells that synthesize insulin. At 4-6 weeks of age, B and T lymphocytes infiltrate the islets (insulinitis) and auto-antibodies against both lymphocytes and β -cells are produced. By 30 weeks, 72 per cent of female and 39 per cent of male mice have diabetes (a sex difference not seen in humans). Disease can be transferred with T cells from diabetic animals and requires both CD4⁺CD8⁻ and CD4⁺CD8⁺ cells; "Type 1 diabetes is therefore a T-cell-dependent and probably a T-cell-mediated disease" (ref. 7).

Immune and autoimmune T-cell responses are provoked by molecular matching of their receptors with peptide antigens bound to polymorphic MHC glycoproteins (see the recent News and Views article by Germain⁸). Consistent with this mechanism is the mapping of diabetes susceptibility genes to the human⁹ and murine¹⁰ MHC. The strongest associations are seen with alleles encoding human HLA-DQ and murine I-A molecules⁷.

NOD mice have a distinctive MHC. Although alleles encoding the I-A α -chain and class I MHC molecules are

identical to those found in BALB/c mice (H-2^d), the I-A β -chain allele is novel and its encoded polypeptide differs by 10-20 residues from other I-A β -chains⁶. NOD mice do not express the second type of class II molecule (I-E) owing to a non-functional I-E α -chain gene, and thus have a single, unique class II MHC molecule (I-A^{NOD}) for the presentation of antigens to CD4⁺CD8⁻ T cells. Previous breeding of a functional I-E α ^d transgene (not integrated in the MHC) into NOD mice showed that expression of I-E^d molecules completely prevented insulinitis and diabetes¹¹. Lund *et al.*¹ confirm this result with a genetically cleaner experiment in which the I-E α ^d transgene is directly introduced into NOD mice. Overall, these findings have led to speculation that certain novel, 'undesirable' antigen-presenting properties of I-A^{NOD} cause the MHC-linked component of NOD diabetes. The three groups writing in this issue of *Nature* collectively assessed the proposition by introducing transgenes encoding other I-A molecules into NOD mice.

Miyazaki *et al.*² and Slattery *et al.*³ provided their NOD mice with I-A^k molecules and found insulinitis was dramatically reduced and that there was no progression to diabetes. The disease susceptibility conferred by I-A^{NOD} is therefore recessive and can be overridden by the presence either of I-E^d (refs 1 and 11) or of another I-A molecule, I-A^k (refs 2 and 3). Thus rather different class II MHC molecules can overcome the diabetes-inducing deficiencies of I-A^{NOD} molecules.

Diabetes and Asp 57

I-A^{NOD} and I-A^k differ by 14 amino acids in the α -chain¹² and 13 in the β -chain⁶. Systematic site-directed mutagenesis is the logical approach for identifying which of these many substitutions are important for disease susceptibility. Miyazaki *et al.*² and Lund *et al.*¹ take small, but calculated, steps in this direction. Of interest is that their mutations concern position 57 of the β -chain, a focus of extraordinary discussion^{5-7,9,13-15}. Underlying the Asp 57 controversy is the long-standing debate on the significance of HLA disease associations.

Numerous human diseases are associated with HLA type¹⁶. Tissue typing was first confined to class I HLA molecules, and in the case of type 1 diabetes susceptibility was initially correlated with HLA-B8 and B15 antigens. Subsequent discovery of class II MHC antigens revealed stronger associations with HLA-DR3 and DR4, class II antigens in linkage disequilibrium with the previously associated

HLA-B alleles⁹. With more precise definition of HLA alleles, it became clear that there was a stronger disease association with HLA-DQ than with HLA-DR, and that the shifting association could again be explained by linkage disequilibrium. Thus for some time the diabetes-susceptibility gene has been moving progressively towards the centromere of human chromosome 6 (see figure), and old HLA hands may be sceptical of claims that this trend has stopped. Such refinement of HLA association with various diseases led to arguments that imprecision in HLA typing obscured the true relationships, and that at the level of the nucleotide sequence systematic differences between the 'same' gene in patients and healthy controls would emerge. Many identical sequences were acquired in attempts to validate this hypothesis, and given the limited insight gained it was with understandable enthusiasm that McDevitt and colleagues embraced correlation of a single amino acid, aspartic acid, at position 57 (Asp 57), with genetic predisposition to diabetes⁵.

In Caucasians, HLA-DQ β -chains having Asp 57 were associated with a dominant resistance to diabetes, whereas DQ β -chains with other amino acids were associated with susceptibility. Adding conviction to this correlation was the presence of Asp 57 in all murine I-A β -chains, except that of the NOD mouse, which has serine⁶. Furthermore, histidine 56 of the I-A^{NOD} β -chain was also distinguished from the proline or serine found in other I-A and all DQ β -chains. These findings implicated His 56 and Ser 57 as the source of the NOD mouse's problems.

Miyazaki *et al.*² assessed the protective effect of transgenic I-A^k molecules with Asp 57 replaced by serine, a clear test of whether Asp 57 is essential for protection. Their result — that just 17 per cent of NOD mice transgenic for Ser 57 I-A^k molecules display insulinitis compared with 83 per cent of control NOD mice — proves that it is not, and in fact the removal of Asp 57 from I-A^k improves this molecule's protective effect.

Lund *et al.*¹ took a complementary approach and made NOD mice transgenic for an I-A β ^{NOD} gene in which codon 56 was changed from encoding histidine to encoding the proline found in I-A^k. It turns out that this single substitution is sufficient to convert I-A^{NOD} into a molecule conferring dominant protection against disease. So Lund *et al.* also prove that Asp 57 is not essential to protect NOD mice from diabetes; their experiment, however, does show that single amino-acid substitutions in the vicinity of residue 57 can profoundly affect the diabetic propensity of an I-A molecule.

Despite discomfiting the dogma, these findings are unlikely to dampen enthu-

siasm for Asp 57, or for this autumn's symposium celebrating Hugh McDevitt's sixtieth birthday, for they reflect the complexity and heterogeneity exposed by study of Asp 57 and diabetes in human populations. The role of HLA-DQ in diabetes and the strength of the Asp-57 correlation have consistently been confirmed^{7,13,14}, and it is now clear that disease susceptibility is correlated with the presence of molecules formed by particular combinations of polymorphic DQ α -chains with polymorphic β -chains. (This conclusion is supported by results of Miyazaki *et al.*² showing that the β -chain of I-A^k protects against NOD diabetes only when it associates with the α chain of I-A^k and not with the α -chains of I-A^d.) So the same HLA-DQ β -chain can affect either disease resistance or susceptibility in combination with different DQ α -chains.

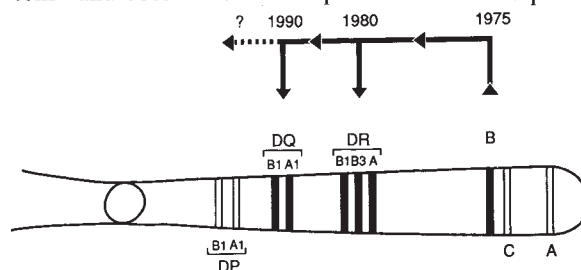
In general, molecules with β -chains containing Asp 57 are more likely to offer resistance, but there are examples where the reverse is true⁷: similarly for disease susceptibility and non-Asp-57-containing β -chains. The emerging picture is one in which each unique HLA-DQ molecule contributes a unique predisposition to disease^{9,15}. Thus some HLA-DQ molecules confer varying degrees of resistance to diabetes, and some seem to be neutral^{7,9}. The details are confusing, but the general principle makes sense if one looks at diabetes as an autoimmune disease, the product of T cells activated by self-peptides bound to MHC molecules.

Mechanisms of diabetes

Distinguishing self from non-self is fundamental to the immune system. For T cells, this discrimination is a dangerous balancing act, swaying between extremes of non-responsiveness and autoimmunity. Their receptors recognize peptides presented by MHC molecules at cell surfaces; interactions between immature T cells and complexes of self-peptides and self-MHC molecules serve both to create a state of tolerance to self and to select a repertoire of mature T cells for responses to foreign antigens. Several mechanisms are involved. In the thymus, where T cells develop, complexes of self-peptides and self-MHC molecules delete one subset of autoreactive clones and select another subset of T-cell clones. The second group leaves the thymus, enters the circulation, and can there encounter complexes of MHC molecules with peptides derived from self-proteins not found in the thymus. T cells that react with such complexes are not destroyed but are tolerated by inactivation (anergy) or suppression by other T cells⁶.

Pancreatic β -cells are highly specialized; they express proteins (including insulin) not found in the thymus and against which potentially reactive, but

anergized or suppressed, T cells probably exist. One idea is that this state of peripheral tolerance is broken as a result of changes in immune response to environmental antigens and microorganisms; evidence for this is the predominant involvement of HLA-DQ molecules in generating antigen-specific suppressor cells¹⁷ and observations that spontaneous



Cartoon of the short arm of chromosome 6, showing the positions of HLA loci that have been linked to susceptibility to diabetes and the approximate date of the associations.

diabetes in NOD mice is increased by keeping the animals pathogen-free and is decreased by a variety of treatments that activate T-cell responses (reviewed in ref. 7).

A second possible mechanism for induction of diabetes is that expression of class II MHC molecules on pancreatic β -cells (where they are not normally expressed) results in presentation of a novel spectrum of MHC-self-peptide complexes for which T-cell tolerance does not exist¹⁸. These complexes become the targets for T-cell autoimmunity, and in last week's *Nature* Roep *et al.*¹⁹ described autoreactive T cells from a diabetic which recognize a peptide, derived from a membrane protein (relative molecular mass 38,000) of the β -cell's secretory granule, presented by HLA-DR1. Although unusual expression of class II MHC in the pancreas of human diabetics¹⁸ and NOD mice²⁰ has been documented, it is uncertain whether it is the cause or an effect of the disease. Unfortunately, transgenic targeting of class II MHC molecules to pancreatic β -cells has failed to resolve this issue. It is, however, of interest that increased expression of class I MHC molecules — the targets for cytotoxic T cells — is seen by Lund *et al.* in the lymphocyte-infiltrated pancreatic islets of NOD mice, and that this is eliminated in non-diabetic transgenic NOD mice¹.

The number and polymorphism of class I and class II HLA genes means that most people have a different 'HLA type' and, in consequence, a different repertoire of T-cell specificities. Although most dramatically demonstrated by the alloreactive T-cell response in the rejection of transplanted tissues, differences in T-cell specificity between HLA-disparate individuals are invariably found in responses to foreign antigens, and a similar pattern has been demonstrated for tolerance to self components²¹. Breaking of self-tolerance (that is, autoimmune disease)

should obey similar rules, implying that clinically similar disease in HLA-incompatible people may arise from T-cell responses that differ in specificity and mechanism.

This thesis is supported by the complex genetics of HLA-linked susceptibility to type 1 diabetes⁹, which are difficult to interpret without an understanding of the immunological disease mechanisms and appreciation of the various levels at which MHC molecules control the T-cell response. Asp 57 has been an invaluable molecular signpost in revealing the importance of HLA-DQ molecules in diabetes, but the simplicity of the finding guaranteed both its condemnation^{9,15} and overinterpretation⁵. Asp 57 was all too readily

removed from its context, namely the three-dimensional structure of the antigen-presenting HLA-DQ molecule, temporarily obscuring the importance of unique α - and β -chain combinations in determining a wide range of predispositions to the disease.

The action of various DQ molecules in disease susceptibility may differ, as suggested by their relative dominance and recessivity⁹. For example, thymic deletion of T cells important for autoimmunity would confer dominant disease resistance to a particular DQ molecule. Alternatively, deletion of T cells providing peripheral tolerance to β -cell antigens would confer dominant susceptibility. A DQ molecule that positively selected such regulatory T cells would confer disease resistance but be recessive to a second DQ molecule that deleted them. Predominating effects for some molecules may be at the level of antigen presentation in β -cells. For example, DQ molecules that fail to pre-

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sent antigens could be neutral or confer a recessive resistance to disease; those that present antigens to effector cells would give dominant susceptibility and those presenting antigens to suppressor T cells, dominant resistance. There may also be weaker contributions from MHC molecules other than HLA-DQ, both to the presentation of antigens in the β -cell and to their effects upon T-cell repertoire.

Human type 1 diabetes may eventually come to be viewed as a heterogeneous

PALAEOANTHROPOLOGY

Lining up the ancestors

Peter Andrews

DURING the past decade many of our ideas about human evolution have been turned upside down, not the least of which concerns the fossil evidence for the origin of the human lineage. A new and relatively complete fossil hominoid skull, described by de Bonis on page 712 of this issue¹, will fuel this debate further. Although the new skull is put forward as a direct human ancestor, thereby implying the presence of the hominid lineage in Greece 9–10 million years ago, I have my doubts about this interpretation. The great interest of the new specimen is in its retention of mainly primitive hominoid characteristics at a stage when fossils of similar age show strong affinities with the orang utan lineage.

Before 1980, fossil evidence for the origin of the human lineage was generally considered to rest with *Ramapithecus*, a Miocene hominoid which lived 12–14 million years ago and which is now considered to be the same as *Sivapithecus*. Since then, evidence from molecular studies^{2,3} and better fossils^{3,4} has shown *Sivapithecus* to be more akin to the orang utan than to the human lineage.

The new skull has been assigned to the species *Ouranopithecus macedoniensis* and comes from a locality of similar age to others in Greece where fossil hominoids have been found⁵. It includes many characters generally agreed to be primitive for the group. For example, the large interorbital distance is likely to be ancestral not just for hominoids but also for catarrhines (Old World monkeys and apes) as a whole³. Other characters such as the shape of the naso-maxillary region appear to share the African ape and

collection of autoimmune diseases having a common outcome in the total destruction of pancreatic β -cells. NOD mice have a condition that falls well within this definition, and its continuing analysis should provide insight into at least a subset of the MHC-related immune mechanisms that contribute to human disease. □

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RATIOS OF CANINE AREA TO FIRST-MOLAR AREA ($\times 100$)

XIR-1 (ref. 1)	94
Female chimpanzees	78–108
Male chimpanzees	114–210
Female gorillas	66–114
Male gorillas	111–185
<i>Proconsul</i> , all measurable specimens	103–162
<i>Afropithecus</i> (?male)	217
<i>Sivapithecus indicus</i>	67–111
<i>S. meteai</i> (?female)	107
<i>Ouranopithecus</i> (?male)	97

human condition⁶, for which the orang utan shares a different set of derived character states with *Sivapithecus*. In still other characters, the new skull differs from all recent hominoid skulls — for example, in the broadly prominent supra-orbital torus, which is similar to that of robust australopithecines although not to most other fossil or modern humans. This character is also similar to that seen in *Dryopithecus*⁷ from the Miocene site at Rudabanya, Hungary, so that the similarity with robust australopithecines is probably convergent.

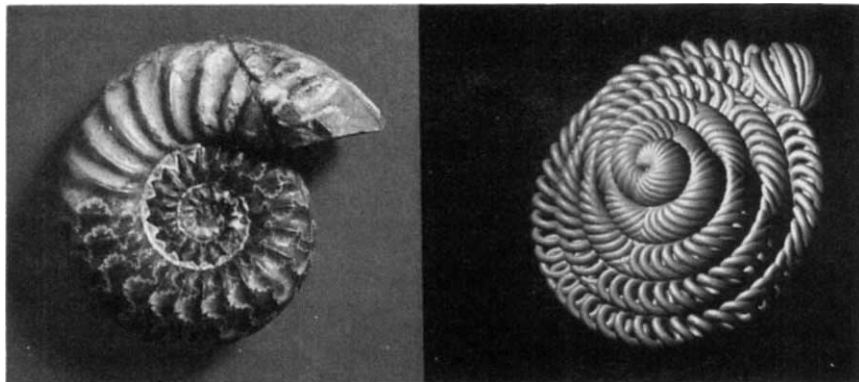
De Bonis lists a final group of characters

present in the new fossil that are shared only with fossil and recent humans. These include reduced canines, the absence of the honing wear facet on the anterior face of the lower third premolar, and the round and swollen molar cusps. The reduction of canines depends very much on the sex of the fossil: if it is male, as de Bonis claims, the canine is small in relation to male canine size in other hominoids, especially in comparison with the canines of the earlier hominoid, *Proconsul*; if female, it is within the range of many recent and fossil hominoids (see table). The difficulty comes with distinguishing normal variation due to sexual differences from real, interspecific differences. The relative size of the canine is also used to distinguish between males and females, and it would be more realistic to identify the new fossil as a female on this basis rather than making claims about canine reduction.

The second character, absence of a honing facet on the lower third premolar, is a corollary of, and dependent on, small canines. As with canine size, honing facets are reduced on females anyway, and this effect cannot be distinguished from any absolute change in honing facet. The round and swollen molar cusps are indistinguishable from those seen in a wide range of fossil hominoids, including *Sivapithecus* (and *Ramapithecus*). This character differs, and is presumably derived, from the ancestral hominoid condition as present in the early Miocene *Proconsul*, but it is more likely to be an ancestral character of great apes and humans because of its association with thick enamel⁸. Recent work on a specimen of *Ouranopithecus macedoniensis* by Lawrence Martin (personal communication) has confirmed the great thickness of the molar enamel of this species.

Most of the characters by which the orang clade can be recognized (listed in ref. 3) are absent in the new fossil. In the middle to late Miocene there were other species like *Lufengpithecus*⁹ in China,

Forms of imagination



Nature (left) meets computer art (right) in a new exhibition of computer 'sculpture' by William Latham at the Natural History Museum in London. The exhibition, which includes interactive 'hands on' displays, opened last week and runs until 16 September.

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Kenyapithecus in Africa, and now *Ouranopithecus* from Greece, all of which have thick enamel, but there is no evidence to indicate how these species might be interrelated. It is not known, for instance, which of them (if any) is most closely related to humans. More detailed descriptions of the new skull may throw some light on this. But it is evident that there was a much greater diversity of

hominoid genera and species in the mid-Miocene than was recognized ten years ago, and as more and better specimens are recovered during the 1990s our understanding of the early stages of human evolution should increase correspondingly. □

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COSMOLOGY

A large-scale puzzle

Virginia Trimble

GEORGE Gamow is supposed to have said that, with five free parameters, a theorist could fit the profile of an elephant. In practice, astrophysicists are not often called upon to do this. But theorists at a recent conference* showed that they can fit a structure almost as complex and unexpected — the large-scale distribution of galaxies in the Universe — with an average of only two parameters per theorist — not, of course, the same two in each case. The observations, long a subject of rumour and colloquium in the astronomical community, first appeared officially in February¹, and were not easily explained by conventional models of galaxy formation². They still are not.

The data comprise several hundred redshifts of galaxies, located in two narrow, cylindrical volumes of space in the directions of the north and south poles of our Galaxy (where interstellar obscuration is smallest). The volumes extend out to redshifts of more than 0.3 in each direction (corresponding to distances of $1,000 h^{-1}$ Mpc, where h is the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$). Previously known nearby clusters and superclusters are recovered, showing that the basic method is sound. But in addition, a plot of numbers of galaxies as a function of redshift displays about seven sharp peaks, spaced fairly uniformly $128 h^{-1}$ Mpc apart. The same length scale appears in the Fourier transform (the power spectrum) of the data and in a plot of pairwise separations of the galaxies in the sample. Galaxies that, according to conventional wisdom, grew out of a gaussian distribution of initial perturbations^{3,4} — even with additional non-random perturbations seeded by cosmic strings or galactic explosions — cannot behave in this way. But it seems that there are other possibilities.

The observations (A.S. Szalay, Eötvös University and Johns Hopkins University) have not changed. Work is progressing, however, on samples in other

directions in space. Smaller numbers of redshifts for galaxies lying about 45° from the Galactic plane (Selected Area 68) and towards the Hercules cluster show less distinct peaks on slightly different scales, also near $100 h^{-1}$ Mpc (which are somewhat dependent on the cosmological model assumed). Curiously, nothing much showed up on larger scales, leading E. Bertschinger (MIT) to suggest that perhaps we are already seeing the largest-scale coherent structures in the Universe — contrary to the impression given by the shallower but wider Center for Astrophysics survey¹.

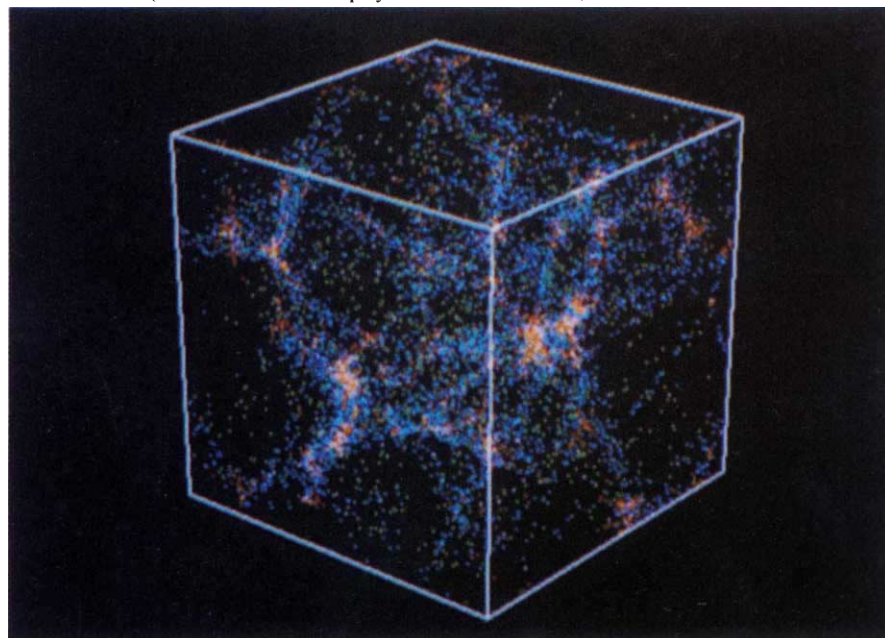
It will be some years before there are large surveys in enough different directions to provide a three-dimensional picture of the Universe. Meanwhile, it is possible to consider the results from the Galactic-pole survey simply as a Fourier peak of a particular amplitude, width and wavenumber — just the sort of feature to inspire a new parameter or two.

L. Kofman (Institute of Astrophysics

and Atmospheric Physics, Tartu) discussed a standard (Harrison–Zel'dovich) power spectrum of initial perturbations that arise from quantum fluctuations in a scalar field whose primary function is to drive inflationary expansion of the Universe⁵. Inflation is a hypothetical brief epoch early in the Universe during which expansion is exponential in time, energy for this being derived from a field for which at present we have no independent evidence. Such expansion could account for the present near-smoothness and near-flatness of the Universe. The simplest possible field is a scalar one, which takes on a single value at each point in spacetime. Kofman showed that a second scalar field could be present, and could be made to yield extra power on a particular scale (50 Mpc in his trial model). The amplitude and wavenumber of this peak can be adjusted to match those of the Galactic-pole data, but the peak is never very sharp on the long-wavelength side.

V. Mukhanov (Institute for Nuclear Research, Moscow) pointed out that more than two scalar fields could easily lead to more than one characteristic clustering length. L. Grischuk (Sternberg State Astronomical Institute, Moscow) meanwhile admitted that his 'squeezed' gravitons (analogous to squeezed states in quantum optics) might leave detectable traces on the spectrum of pregalactic primordial fluctuations. (A smooth sea of gravitons should pervade the Universe in much the same way as the sea of 3-K photons.) But Grischuk disclaimed responsibility for the 128-Mpc periodicity on the grounds that he is describing four-, not three-, dimensional spacetime.

V. Icke (Sterrewacht, Leiden) address-



A cube of the Universe modelled by means of the Voronoi process. Shown are 10,000 galaxies initially distributed at random in a cube with sides of ~ 80 Mpc. Galaxies are colour-coded: white, galaxies in Voronoi cells (voids); green, galaxies in Voronoi walls; blue, in filaments; red, in nodes ('Abell clusters'). Figure by V. Icke (Leiden Observatory, The Netherlands).

* Workshop on the Legacy of Ya. B. Zel'dovich for Astrophysics and Cosmology, 2–7 May 1990, University of Kansas in Lawrence, USA, held under a program for cooperation in basic research between the Soviet Academy of Sciences and the US National Science Foundation.

sed large-scale structure as a geometrical rather than a dynamical problem. He concentrated on the voids between the galaxies, because, he said, that is where most of the Universe is. The underlying idea is that underdense regions of the Universe expand and attempt to become more spherical; eventually they fill space in a nonrandom way, called Voronoi tessellation⁷ and looking like soap bubbles squeezed together. Galaxies occupy the walls, edges and vertices of the resulting cells nonrandomly, so that the topology is more like that of a sponge than a honeycomb. A pencil-beam survey through such a universe will intersect successive cell walls, predominantly those of the larger cells, and so is likely to yield periodic peaks in the numbers of galaxies as a function of redshift.

A sequence of two-dimensional Voronoi tessellations described by P. Coles (University of Sussex) demonstrated that the geometry has a 5–10 per cent chance of producing peaks in the galaxy spacing as narrow as those seen. In addition to the assumption about how voids grow, there is a second free parameter — the initial density of void centres. This must be chosen to make the final number of vertices equal to the number of rich clusters of galaxies in real catalogues. After normalizing to the Abell catalogue, Coles found that the spacing of peaks in number of galaxies versus distance comes out to be $137 h^{-1} \text{ Mpc}$.

Somewhat less regular structure (analogous to Johnson–Mehl tessellation⁸) arises when the evolution of perturbations to the primordial density distribution is followed far into the nonlinear regime by means of equations that describe the gravitational potential distribution (rather than galaxies or voids directly). Its inventors call this the adhesion model¹⁰ because of the effect of ‘viscosity’ when particle trajectories attempt to intersect. It is at about this point that the Zel’dovich approximation⁹ usually breaks down. As described by S. Shandarin (University of Kansas and Institute for Physical Problems, Moscow) and D. Pogosyan (Institute of Astrophysics, Tartu), the model reproduces many of the properties of real galaxy distributions but not a regular spacing of numbers versus redshift.

The prize for best numerical coincidence went to A. Heavens (University of Edinburgh), whose fine-tuned version of an earlier model¹¹ had a peak in the two-body correlation function at precisely 127 Mpc. The model assumes a universe with 20 per cent of the closure density (all in the form of ordinary matter) and incorporates fluctuations with constant space-time curvature (isocurvature fluctuations). Unfortunately the peak occurs after the correlation function has already dropped below zero at $55 h^{-1} \text{ Mpc}$, so that the peak in fact represents a ‘deficit of anticorrelations’.

Do any of these schemes approach a complete description of the real large-scale structure of the Universe? The

more apposite elephant analogy here may be that of the five blind sages examining its head, tail, legs, trunk and ears and drawing appropriate conclusions. Virtually all the speakers made some reference to this incompleteness aspect of their calculations. The important point, however, is not whether we have fully understood the origin of the structures reported but whether it can be shown that they are physically impossible. Clearly they are not. Puzzling, perhaps improbable — but not impossible. □

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PROTEIN MICROSCOPY

Bacteriorhodopsin — at last!

D.L.D. Caspar

HISTORY is made by Richard Henderson and his colleagues in Cambridge, Berlin and Berkeley with their report¹ this week of an atomic model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. This is a result that has been anticipated for 15 years. Ever since 1975, when Henderson and Unwin² demonstrated that a distinct image of the seven transmembrane α -helical rods in bacteriorhodopsin could be resolved by Fourier-averaging fuzzy electron-micrographs of unstained purple-membrane lattices, it has been evident that the atomic structure of membrane proteins could be determined by electron crystallography³.

Why has it taken so long to solve the structure of the first intact membrane-embedded protein? And why did Henderson bother with electrons when protein crystallographers have been so successful at solving complicated structures with X rays? In many ways, the history of protein electron microscopy recapitulates that of protein X-ray crystallography in Cambridge, which started in 1934 when Bernal and Hodgkin obtained the first high-resolution X-ray diffraction patterns. In 1953 Perutz found the key to unscrambling these patterns by using the method of isomorphous replacement to determine the phases; five years later, Kendrew and colleagues solved the structure of myoglobin, producing the first atomic model of a protein structure. In his work, Henderson used the method of three-dimensional reconstruction from the Fourier transforms of tilted images developed by DeRosier and Klug in 1968. But the problems of obtaining interpretable high-resolution electron images of tilted, flat, periodic membrane specimens have proved more formidable than expected in 1975. Henderson’s

achievements in making protein microscopy a practical science rank with those of his distinguished predecessors who invented protein crystallography.

The value of using electrons to solve the structure of bacteriorhodopsin in a two-dimensional crystal is explained by the intrinsic properties of the purple membrane. This rugged, light-driven proton-pumping membrane lattice was first isolated from *Halobacterium halobium* 22 years ago by Stoekenius; it has since become an object of central importance in membrane biology. A wealth of data has accumulated about the orientation, location and interactions of the retinal chromophore within bacteriorhodopsin, and the mechanism of proton translocation across the membrane. In particular, by analysing many site-directed mutants, Khorana⁴ has established the role of key amino-acid residues. But direct information about the three-dimensional structure is essential for understanding the functional organization, and at present only electrons can provide this information about bacteriorhodopsin in intact membranes.

The map computed by Henderson *et al.*, which has a resolution of 3.5 Å parallel to the membrane plane but lower vertically, is sufficiently detailed for them to define an atomic model, making use of the information about specific amino-acid interactions and chromophore structure from other studies. The folding of the polypeptide chain follows the path predicted a decade ago⁵, and a plausible mechanism for the proton translocation emerges from the structural model which is consistent with a great deal of other data¹ (see figure). Relating the structure and the function of bacteriorhodopsin has special interest because of the way in which the structure was solved using

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high-resolution electron microscopy.

To compute an averaged three-dimensional map of bacteriorhodopsin, Henderson *et al.* first obtained two-dimensional electron images of variously tilted specimens to determine the phases of the independently measured three-dimensional electron diffraction data. Amplitudes of the Fourier coefficients of the structure can be measured from the diffraction pattern more accurately than they can be calculated from the images after correction for the defocus. Because the transforms of the best images correlate well with the measured diffraction data, this method of phasing is reliable. Isomorphous replacement is not a useful method for phasing the electron diffraction data from two-dimensional protein crystals because the scattering contrast of heavy atoms is low for electrons and the noise level in the patterns is relatively high. Even though the higher-resolution detail in the best Fourier-averaged purple-membrane images is weaker than it should be with a perfect microscope⁶, the resolution is adequate to reveal the molecular structure.

The main problems in protein microscopy are (1) preparing flat, well ordered two-dimensional crystals suitable for high-resolution image averaging, (2) avoiding radiation damage, and (3) designing a microscope that images high-resolution detail. These problems are interrelated, because recording high-resolution detail requires correction for intrinsic specimen disorder and minimization of beam-induced motion. Glaeser recognized the limitations on specimen lifetime due to radiation damage over 20 years ago, and he has pioneered developments of cold stages and electron optics suitable for high-resolution imaging of biological specimens⁷ that have helped to make Henderson's new microscopy a reality.

The best native purple-membrane image so far obtained came from an untilted specimen with the liquid-helium-temperature microscope in Berlin designed by Dietrich *et al.*⁸; and the best image of a tilted specimen was obtained at Berkeley using the microscope with a

field-emission gun and computer-controlled spot scan developed by Downing and Glaeser⁹ which operates at a relatively warm temperature of 150 K. The spot-scan procedure^{6,9} reduces the blurring caused by beam-induced movement,

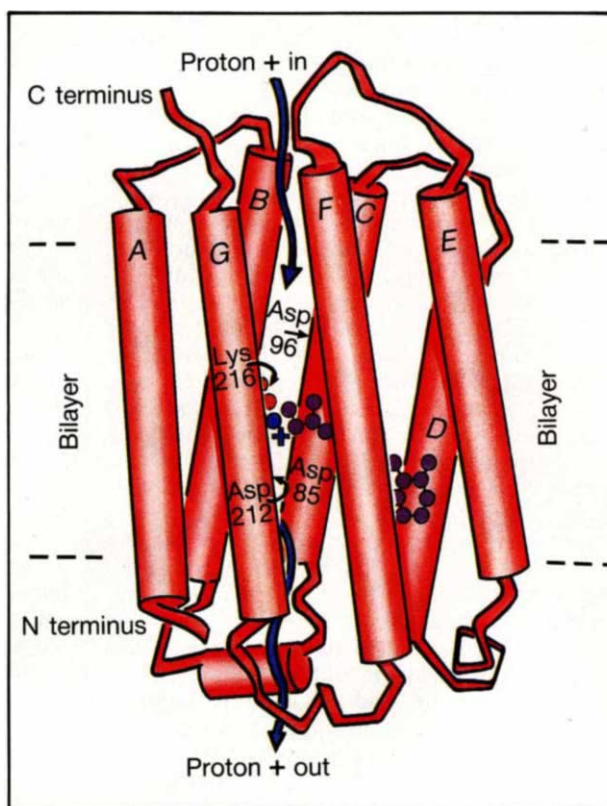
microscope combining all these features with computer control of all functions and an operating voltage of 200–400 kV. The collection of high-resolution protein-image data could then be automated – perhaps like the collection of protein crystal diffraction data with a high-intensity X-ray source and a computer-controlled area detector.

As Perutz has pointed out, protein crystallography gradually progressed from ingenious puzzle-solving to almost blindfold automation. Protein microscopy is still at the puzzle-solving stage, but it is no longer the fantasy it may once have seemed to be. In his biography *What Mad Pursuit*, Francis Crick recalled the days when other crystallographers believed that protein crystallography was hopeless. He noted "the curious mental attitude of scientists working on 'hopeless' subjects...they are all buoyed up by irrepressible optimism.... Anyone without such optimism simply leaves the field. So...workers in subjects in which the prize is big but the prospects of success very small always appear very optimistic. And this in spite of the fact that, although plenty appears to be going on, they never seem to get appreciably nearer their goal". Henderson has now attained his prime goal after 15 years of "irrepressible optimism".

What next for protein microscopy? The technique has already been applied to determine a 3.5-Å-resolution projection image of a bacterial porin membrane crystal¹⁰, and the three-dimensional 3-Å-resolution electron diffraction data that have recently been collected are yielding an interpretable map. Monolayer crystals of the light-harvesting complex¹¹ diffract to high resolution and may also soon be imaged in three dimensions with electrons. Thin crystals of the Ca²⁺-dependent

ATPase from the sarcoplasmic reticulum membrane¹² are a promising system, as are other membrane proteins that form planar arrays with lipids, and some soluble proteins that crystallize as thin plates. Richard Henderson foresees the day when improved microscopes will turn the technique that he has developed into a routine method for solving the atomic structure of complex thin biological specimens, including noncrystalline protein assemblies. □

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Bacteriorhodopsin proton pump. The membrane retinal chromophore (coloured purple) is in the middle of the schematically represented bacteriorhodopsin molecule whose atomic structure has been determined by Henderson *et al.*¹. A–G designate the seven transbilayer α -helical rods, of length 30–40 Å, connected in alphabetical sequence by the segments alternately at the cytoplasmic (top) and extracellular (bottom) surfaces of the purple membrane. Residues from the helices A, B, C, F and G form the proton channel and interact with the chromophore which is linked to Lys 216 on G. Ionized Asp residues 85 on C and 212 on G, located in the hydrophilic channel extending down from the retinal, are involved in transporting the proton from the Schiff-base nitrogen to the outside after photoisomerization. Protonated Asp 96 on C, located in the hydrophobic channel above the retinal, provides the proton that recharges the Schiff base. Reprotonation of Asp 96 from the cytoplasm completes the photocycle.

which is most troublesome perpendicular to the surface for highly tilted specimens. The field-emission gun gives a beam with high spatial coherence which improves the resolution for defocused regions of the image. At the temperature of liquid helium, radiation damage is minimized. Henderson concludes that the time is ripe for the development of a

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Detecting the invisible

Carl D. Murray

IN 1980 the cameras on board Voyager 1 showed the F ring of Saturn to be narrow and dust-rich, and to contain several peculiar features — for example, radial discontinuities (kinks), clumps of ring material and an apparent twisted structure (braids). Two small satellites, Pandora and Prometheus, were discovered, one on each side of the ring. The torques from these moons are generally believed to be responsible for 'shepherding' the ring material, preventing ring spreading. A new analysis of Voyager images by Kolvoord *et al.*, reported on page 695 of this issue¹, now reveals the tell-tale gravitational signature of Prometheus in the structure of the ring, which may be the cause of its braiding. The analysis also produces evidence for a third satellite at a distance of 1,180 km from the ring, thereby providing an example of our ability to detect such objects even if they cannot yet be imaged directly.

Dermott² showed that, as well as shepherding ring material, satellites should have the more obvious effect of inducing waves as they pass by. On encountering a satellite, ring particles initially on circular orbits would move to eccentric orbits, all with the same eccentricity but with slightly different phases, giving the appearance of a wave on the ring. The wave would eventually be damped through inter-particle collisions, which make the orbits more circular again. The wavelength of such an induced wave is a function of the distance between the ring and the satellite, and the amplitude is a function both of this distance and of the mass of the satellite. This mechanism was thought to be at least partly responsible for the braided appearance of the ring, through the interactions of the waves produced by both of the shepherding satellites. Prometheus' crossing of the F ring every 18 years³ should also have dramatic consequences for the ring's structure.

Kolvoord *et al.* report direct evidence of the wave mechanism at work. Although they find no signature from the smaller and more distant satellite Pandora, one set of periodicities in their F-ring images does correspond to that which would be expected from Prometheus. They also claim to have detected the signature of at least one other satellite, although this evidence is less conclusive. A full explanation of all the structure seen in the highest-resolution images (not included in the data set of Kolvoord *et al.*) will probably require an understanding of the effect of small (radius less than 10 km) satellites orbiting within the ring itself. Although the detailed structure of the F ring is complicated, it may prove possible

to explain it in terms of the simple gravitational effect of a few nearby satellites.

As further sets of high-resolution images of orbiting material will not be obtained for several years, the detection of satellites and rings by indirect methods has become a necessity. This approach has already met with some success for Saturn. The ring occultation observations made by Voyager revealed very few real gaps, as opposed to density fluctuations, in Saturn's rings. One of the gaps, the 325-km-wide Encke division in the broad A ring, has an unusual ringlet, and Cuzzi and Scargle⁴ have shown that the edges of the gap have a wave-like appearance at some longitudes. These waves have a much more regular appearance than the braiding seen in the F ring, and Cuzzi and Scargle concluded that they are strongly suggestive of a satellite of 10-km radius orbiting near the centre of the gap. Such a satellite would have been at the threshold of detection by the Voyager cameras. Preliminary studies⁵ of the Keeler gap (width ~38 km) near the outer edge of the A ring suggest that the width in fact varies by about 6 km, and that the wavelength of these fluctuations varies with longitude. Again, one or more small satellites orbiting nearby have been invoked to account for the observations.

The rings of Uranus were discovered serendipitously by ground-based occultation in 1977. Even today, most of our knowledge of the uranian ring system has been derived from occultation studies rather than from direct imaging. The first

ECOLOGY

Fruits for the taking

Peter D. Moore

GETTING from one place to another can be a serious problem for rooted plants. But being the first to arrive at a suitable location gives undoubted competitive advantages, hence the wide range of fruit-dispersal mechanisms found in the plant world. The structural mechanics of plumed fruits provides a seemingly unlimited source of data for aerodynamic analysis and the assessment of transport efficiencies¹, but the evolutionary relationship between fruits and their vertebrate dispersal vectors has stimulated even more ecological research projects. The search continues for a general relationship between fruit size, morphology and chemistry, and the bills, gapes, jaws, maws and guts of their bird and mammal consumers. Some fairly broad conclusions are beginning to emerge.

such study of material in orbit around Neptune took place in 1981⁶. Although occultation events in subsequent years were attributed to the presence of incomplete rings or 'arcs' around the planet, the opaque nature of the 1981 event prompted its observers to maintain that they had detected a satellite. The Voyager flyby of Neptune in August 1989 led to the discovery of six new satellites, one of which (temporarily named 1989N2) was of the right size (radius ≈ 100 km) and in the right orbit to explain the 1981 event⁷, confirming that the satellite had indeed been first discovered by occultation.

Perhaps the most unexpected source of information on the locations of rings and satellites consists of charged-particle experiments. Rings and satellites create 'shadows' or 'wakes' in the plasma environment around planets. Observations by Pioneer 11 at Saturn showed reductions in the particle flux at various locations; some could be identified with the orbits of known satellites and rings, but others were attributed to material not previously known. This led to the discovery of Saturn's G ring and confirmed the existence of the satellite Janus, both of which were subsequently imaged by the Voyager spacecraft. □

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Generally, fruit-eating birds are large and have a relatively wide gape, as has been demonstrated in temperate areas², the Mediterranean region³ and tropical forests⁴. For their area of study (near Montpellier in France), Debussche and Isenmann³ report the results of an eight-year analysis of vertebrate involvement in fruit dispersal and show that there is a strong positive correlation between the gape size of birds and the volume of the fruits they feed upon. There is a similarly positive relationship between the disperser weight (both birds and mammals) and fruit volume.

The second finding is reminiscent of the relationship between predators and their prey, but there are marked differences in the case of fruit-eating, as Debussche and Isenmann point out. In the case of fruit, it

is advantageous to be eaten, so small fruits may be conspicuously coloured or hang together in clumps and thus become more evident and attractive to larger feeders. Small animals, on the other hand, may consume only part of a larger fruit and yet pick up seeds and disperse them: so black-caps can disperse figs even though they cannot swallow the entire fruit. Rather surprisingly, mammals seem to avoid eating protein-rich and lipid-rich fruits, perhaps because such fruits often contain toxins or volatile compounds.

Debussche and Isenmann found no overall differences between the colour preferences of birds and mammals; on the other hand, tropical forest research¹ suggests that birds prefer red-purple fruits whereas monkeys prefer red, orange and yellow. In the Mediterranean the mammals may use olfactory rather than visual methods of fruit foraging.

The question of coevolution (the "reciprocal evolutionary change in interacting species", as Thompson² defines it) is inevitably somewhat speculative in the case of fruit-disperser relationships, because direct evidence in the form of parallel series of fruit and disperser fossils is scarce. Some birds, like the waxwing, are almost completely frugivorous and are clearly committed to this diet; others, like the starling, will take fruit in its modern urban habitat, but may not have done so as often before the rise of cities. Debussche and Isenmann propose that, among the 20 birds and five mammals that feed upon fruit in their area of study, seven species (including Sardinian warbler, blackcap and stone marten) are responsible for over 90 per cent of the fruit dispersal, and that these have been the main source of evolutionary selection on the fruits.

This view, however, neglects any consideration of the time element. Changing climates and hence vegetation patterns and assemblages in the past would have exposed both fruits and dispersers to very different conditions and selective pressures. The historical aspect of the interaction has been examined by Milton, Siegfried and Dean³ in their studies of the arid Karoo rangelands of South Africa. Mammals may disperse fruits without consuming them, as in the case of adhesive fruits equipped with spines, hooks and barbs, and the frequency of plant species with this sort of mechanism in a vegetation type may be taken to reflect something of its grazing history. In the arid south-western Karoo, for example, there is a lack of plants showing adhesive dispersal and vegetative spines associated with defence against browsers and grazers. Evidently, this vegetation has not been subjected to selective pressures from large mammals, so has had no need of defensive spines and no opportunity to disperse by sticky fruits, unlike the northern Karoo where ungulates are abundant, and trees

have thorns and grasses have barbed fruits. In the south-west, the only places where plants with such features occur are along the lines of watercourses where plant productivity is higher and some penetration of large grazers has occurred, and also in locations where domestic Merino sheep have been responsible for their introduction. It is impossible to say how long this situation has existed, but the earliest written descriptions of the area from the eighteenth century suggest that even then game animals were scarce in the south-west Karoo. Its vegetation seems to have evolved without being affected by large browsers.

The longer-term relationships between fruits and dispersers are still unknown, but perhaps associations developed and held

ARCHAEOASTRONOMY

The Moon and the crucifixion

Clive Ruggles

THERE are just enough tentative threads of evidence, Biblical and historical, to hold out the hope of astronomically dating the crucifixion of Jesus Christ. Isaac Newton came up with 23 April AD 34, although this date has generally fallen from favour on historical grounds. The possibilities have become narrowed down to two clear favourites, 7 April AD 30 (ref. 1) and 3 April AD 33 (ref. 2), with the balance tipped in favour of the latter because of the occurrence on that day of a lunar eclipse³. But the arguments depend critically on assumptions made about the visibility of the Moon to the naked eye. A new assessment of these assumptions⁴ seems now to leave the question wide open again.

The Biblical data are straightforward. The crucifixion took place between AD 26 and AD 36, during the period when Pontius Pilate was procurator of Judaea, and there are good reasons for placing the event in the middle years — say, from AD 29 to AD 33. All four gospels state clearly that the crucifixion occurred on the afternoon before the beginning of the sabbath, that is (in terms of the Julian calendar), on a Friday afternoon. There is some confusion as to whether it happened on the day before or on the day of the Passover feast, corresponding to the Jewish dates 14 Nisan and 15 Nisan.

Problems arise in translating these constraints into a Julian date, largely because of the empirical nature of Jewish calendars. First, Jewish months are lunar synodic months: the beginning of the new month is determined nominally by detecting the first visibility of the lunar crescent in the evening sky after the new Moon. In the first century AD the determination was based strictly on observation. Second, there are twelve months in the

together even through the changing patterns of climate and vegetation of the Pleistocene. As ever, the human influence, this time through sheep and starlings, is now making the picture more complex. □

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Jewish year. At the time of Christ, the requirement that Passover should fall after the vernal equinox⁵ was used as a criterion for inserting an intercalary month every two to three years, to keep the lunar calendar in step with the solar year. But we do not know how (and hence how accurately) the vernal equinox was itself determined. Finally, other criteria could also affect the insertion of a 'leap' month. First fruits and sacrificial lambs had to be ready for Passover: if this looked unlikely for any reason (such as unusually bad weather), an intercalary month might be decreed ahead of Nisan.

In each of the candidate years, the critical question is whether 14 Nisan or 15 Nisan fell on a Thursday-Friday (Jewish days run from sunset to sunset). Problems with intercalary months mean that, in about one year in two, two possible synodic months need to be considered as candidates for Nisan. But the central problem is that of determining 1 Nisan from estimates of lunar visibility. Most previous analyses have used simple empirical algorithms based on small sets of observational data, obtained in a limited range of locations and under a restricted range of observing conditions.

The new approach⁴ by Schaefer is

CANDIDATE DATES FOR THE CRUCIFIXION BETWEEN AD 27 AND AD 34				
Candidate for 1 Nisan	<i>R</i>	14 Nisan	15 Nisan	
27–28 Mar AD 27	0.2±0.4		10–11 Apr	
28–29 Mar AD 27	?		10–11 Apr	
24–25 Mar AD 30	2.6±0.2		6–7 Apr	
20–21 Mar AD 33	2.0±0.2		2–3 Apr	
8–9 Apr AD 34	2.1±0.2		22–23 Apr	

Dates are based on Schaefer's data⁴. A date is taken to be a candidate for 1 Nisan if the visibility parameter (*R*) is at least 0.0 and that of the previous day is no greater than 1.0. Entries are included only if the resulting 14 Nisan or 15 Nisan falls on a Thursday-Friday. An *R* value for 28 March AD 27 is not given by Schaefer, but will be well above 2.0.

based on theoretical modelling incorporating various physical, meteorological and physiological effects^{6,7} backed up by extensive empirical tests⁸. Schaefer models atmospheric extinction as a combination of Rayleigh scattering from common gas molecules, absorption by stratospheric ozone and Mie scattering from aerosols. The model produces an estimate of the minimum brightness of the Moon required for 50 per cent of adults with average eyesight to be able to distinguish it against the twilight sky at the optimal time on the evening in question.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Christ on the cross — can we date the event?

Schaefer defines a 'visibility parameter' R as the logarithm of the ratio of the apparent brightness of the Moon to this minimum. Values of R between 0 and 1 are taken to imply that the Moon might have been observable with difficulty; values above 2 are taken to indicate that it would have been observable with ease. The standard error in R expresses uncertainties in the observing conditions. Schaefer's data can be used to generate a list of candidate dates for the crucifixion (see table). These agree completely with some earlier analyses⁹, and when taken together with historical arguments they do not challenge the role of 7 April AD 30 and 3 April AD 33 as clear favourites.

Closer analysis, however, casts severe doubt upon the significance of the lunar eclipse on 3 April AD 33. An eclipsed moon is blood red, and there is Biblical evidence of the Moon appearing like blood on the day of the crucifixion, particularly in Joel's prophecy (Joel 2:31), quoted by Peter (Acts 2:20), that the moon "would be turned to blood before that great and notable day of the Lord comes", a passage that can be interpreted as referring to an event at the time of Christ's death, before the resurrection⁹.

But the eclipse on 3 April AD 33 was only partial. When the umbra covers only a small fraction of the Moon's disk, its blood-red colour is swamped by the far brighter remainder of the Moon and is

unobservable. Furthermore, the Moon was actually already in the final stages of the eclipse when it rose in Jerusalem. The time at which the Moon left the umbra can be determined only to an accuracy of a few minutes, because of cumulative uncertainties in the relationship between the Moon's orbit about the Earth and the Earth's rotation about the Sun, but Schaefer's analysis indicates that the rising moon would first have become visible when at most one per cent of its disk was still in the umbra. Even an experienced observer would be highly unlikely to spot the umbral eclipse. Any reddish colour obvious to all observers as the Moon rose could have been due only to atmospheric filtering at low altitude, an effect that can be seen at any moonrise.

This example draws attention to the importance of theoretical models concerning the observational capabilities of the naked eye. Such studies have considerable significance for archaeoastronomy and the early history of astronomy. For example, many of the high-precision lunar horizon observations postulated by Thom to have been undertaken by Neolithic and Bronze Age Britons¹⁰ are found not to be feasible when the variable effects of refraction are taken into account; similarly, many postulated alignments of megalithic structures upon rising and setting stars¹¹ may be discounted on the basis of uncertainties in the extinction angle¹².

For the dating of the crucifixion, however, the implications are not quite so significant. Given the assumption that the Jewish sabbath has fallen unfailingly on every seventh day since Biblical times, there seems no reason, on present evidence, to cast the net wider than the two dates that have been favoured for years. But the evidence that the eclipse of 3 April AD 33 was not observable implies that the choice between these two candidates must once again be weighed equally, at least on astronomical grounds. □

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The logic of love

TRADITIONAL courtship, says Daedalus, is a collective attempt to pair off society's men and women into the thermodynamically most stable set of couples. Partnerships form, dissolve and exchange, each set of couples a slight improvement on the previous one, except for the occasional disaster which has to be written off as experience. Sooner or later the pairing seems optimal enough to be formalized into a set of marriages. Sadly, this formalization is increasingly premature, and the global optimization continues painfully with further reshuffles in the form of divorces and remarriages.

The whole process reminds Daedalus of the mathematical trick of optimizing a big, multi-solution problem by 'iterative annealing'. Having guessed an initial solution, you carry out a sequence of small random changes governed by a fake 'temperature'. If a small change improves the solution, you accept it. If it doesn't, you accept it with a probability related to the temperature — a cunning trick for 'climbing out' of purely local optima. Keep doing this, while slowly lowering the temperature, until no further anti-optimal climbing can occur. You should then have reached, not necessarily the absolute optimum, but certainly a very good one.

So Daedalus presents Pairwise, his new matrimonial computer program. Atom-like, every client in the program is attracted to each of his or her potential partners by some assumed bond-energy, large or small. In real life (as opposed to atomic physics), there is no requirement for this attraction to be reciprocated. So, since the stability of a couple is determined by the less satisfied partner, the effective bond-energy of any 'pair-molecule' is the lesser of their two attraction-energies. The initial high temperature of the assembly rapidly dissociates the more unstable pairs for recoupling. From this torrid and unremitting promiscuity, the assembly is slowly annealed down to a glacial monogamy. The pairing that finally 'crystallizes out' is thermodynamically stable. Everyone has their best feasible choice.

DREADCO sociologists are keen to try Pairwise out on real people. They plan to ask each marriageable inhabitant of a given community to judge the attractiveness of all possible mates. From these 'bond-energies' Pairwise will find the ideal pairing, and the sociologists will wait to see how accurately it comes true. An even better test bed would be a complex knot of desperate marriages in the course of mutual re-permutation, but such convenient circumstances rarely occur outside the pages of modern novels. As a first step, DREADCO is offering a pairing service for discos, parties and singles bars, to cut out the usual covert manoeuvrings. David Jones

Of mice and men?

SIR—Is it true that parents' exposure to radiation and chemicals causes tumours in their children¹? Although my experiments with mice anticipated the suggestion that parental exposure to radiation and chemicals could induce heritable tumours in the next generation^{2,4}, this finding has not been supported by the large-scale epidemiological survey⁵ of the children of atom-bomb survivors in Hiroshima and Nagasaki⁶. Gardner *et al.*¹ recorded a risk of leukaemias (mostly acute lymphatic leukaemia) six to eight times higher than normal in the children of fathers who were employed at the Sellafield nuclear reprocessing plant and who had been exposed to more than 100 milliSieverts (mSv) (as external doses) before conception, although total doses were about a quarter of that in Hiroshima and Nagasaki (see table).

The most puzzling problem is the difference in the epidemiological surveys between Sellafield and Hiroshima/Nagasaki. I believe that my mouse experiments can help to reconcile the difference between the two population studies, assuming that the Sellafield results are in fact correct and not simply due to statistical artefacts. I propose three main reasons for the difference in the human studies: different germ-cell susceptibility to leukaemia-causing mutations in Japanese and English people; different germ-cell stages exposed in the two populations; and different postnatal tumour-promoting environments.

In the first case, I have shown^{3,4} that there is no increase of leukaemia in offspring of the ICR strain of mouse derived from spermatogonia that had been acutely dosed with X-radiation (see table). On the other hand, in the N5 strain, single acute irradiation to the spermatogonia induces about a 10 times greater incidence of acute lymphocytic leukaemia in the offspring than in unirradiated controls, and a high incidence of leukaemias persists in sub-

sequent generations⁷. The large difference in leukaemia incidence in the offspring between the two strains reflects the difference in genetic predisposition to leukaemia induction by radiation. Such a difference could account for the different population responses of Sellafield and Hiroshima/Nagasaki. Lung cancer, for example, developed at a seven-times-higher incidence in white uranium miners than in non-white miners in the United States at equal doses, although not by germinal exposure⁸. Nevertheless, there is a surprisingly large (10 times or more) difference in the doubling doses in the mouse and Sellafield human studies. (The lower the doubling dose, the greater the radiosensitivity, see table). This disparity could result from undetermined doses of either neutron exposure or internally incorporated radionuclides and chemicals in the Sellafield study, which should be taken into account in the estimation of genetic risk.

Second, the extremely high risk of childhood leukaemia from the father's exposure during the 6 months before conception¹ suggests that the postmeiotic sperm is more sensitive to radiation than spermatogonia. This finding is well supported by the mouse experiments; although leukaemia was not induced in ICR mouse offspring by spermatogonial exposure of males, a two- to threefold increase in the incidence of leukaemia was induced by the X-ray exposure of the spermatozoa and spermatids. Similarly, higher mutational sensitivity of postmeiotic sperm has been observed in mice by the specific loci method⁹ and chromosome rearrangement studies¹⁰. The negative results in Hiroshima and Nagasaki which involved both spermatogonial exposure (in males) and immature oocytes exposure (of females) can be explained by the reduced sensitivities to radiation of these stages compared with sperm and spermatids, in addition to the

possible difference in genetic predisposition in the two populations.

Third, some studies at Sellafield have revealed an unusually high contamination of workers' homes with radioactive materials¹¹. Children of irradiated fathers might be sensitive to leukaemia induction by postnatal radiation exposure and other environmental factors. The mouse studies have shown that preconceptionally irradiated ICR mice show persistent hypersensitivity to tumorigenesis when subsequently exposed postnatally to tumour-promoting agents, developing clusters of tumours^{12,13}.

Although leukaemia and other childhood cancers have not increased in the children of atom-bomb survivors in Hiroshima and Nagasaki, other types of cancers, especially adult-type cancers, could increase as this population reaches cancer-prone age. A recent epidemiological survey in Hiroshima and Nagasaki revealed a higher risk of adult-type cancers (but not leukaemia) in individuals of 40 years and older, exposed *in utero* to atomic radiation¹⁴. This was also anticipated by the mouse experiments^{13,15,16}. Parental exposure to radiation does not necessarily increase the incidence of specific childhood human cancers such as retinoblastoma and Wilms' tumour.

There are no similar childhood tumours in mice, and radiation-induced germline alteration (mutation) causing tumours suggests that heritable changes elevate (for the most part) the incidence of various kinds of tumours commonly observed in mice^{3,4,7,13}. Such changes might be concerned with an array of genes modifying immunological, biochemical and physiological conditions which can lead to the increase in tumour incidence. Thus, it will be important to follow all the human subjects throughout their lives to determine whether the mouse studies can predict the human response.

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LEUKAEMIA IN THE F₁ OFFSPRING AFTER PATERNAL EXPOSURE TO RADIATION IN MAN AND MICE

	Dose (mSv)	Relative risk	Doubling dose (mSv)	Rate mSv ⁻¹ (× 10 ⁶)
Sellafield				
All stages of spermatogenesis	≥ 100	3	≥ 33	
Postgonia*	≥ 10	4	≥ 2.5	
Hiroshima				
Spermatogonia	435	1	—	
Mouse				
ICR				
Spermatogonia	360–5,040	1	—	0
Spermatozoa and spermatids	360–5,040	1.9–3.2	450	2.3
N5				
Spermatogonia	5,040	9.6	260	7.0

Relative risk of leukaemia at Sellafield was estimated by subtracting the risk value at conception from those before conception¹. Doubling doses of mice were calculated as a dominant trait^{7,15}. For humans, doses were simply divided by relative risk to estimate doubling doses, because the mothers could also have been exposed to radionuclides via house dust and through their partners^{1,11}.

*Fathers' exposure during the 6 months before conception indicates mainly post-spermatogonial exposure, but includes some spermatogonial exposure.

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Different views

SIR—In his enthusiasm for ESO's adaptive optics prototype system (*Nature* **341**, 675; 1989), S. Dickman does not mention a basic physical constraint that severely restricts its use at visible wavelengths. This is the fact that the maximum diameter in which the wavefront from a star is not disturbed by turbulences in the Earth's atmosphere, that Fried's parameter r_0 , shrinks considerably with the wavelength (r_0 is proportional to the wavelength's 6/5th power).

As J. M. Beckers and F. Merkle have clarified (ESO *Tech. Preprint* No. 3, July 1989), "both the wavefront sensor and the adaptive mirror need to have sufficient spatial and temporal resolution to resolve the significant wavefront spatial and temporal variation. The former are of the magnitude of the Fried's parameter r_0 . This parameter increases with wavelength from $r_0 = 10$ cm in the visible for 1 arcsec seeing to $r_0 = 60$ cm in the K band ($2.2 \mu\text{m}$) and $r_0 = 380$ cm at $10 \mu\text{m}$. The durations of the temporal variations also increase proportional to the Fried's parameter so that the number of photons available for the wavefront sensing increases as the

cube of this parameter."

That explains why the adaptive optics prototype system yielded perfect image improvement in the infrared beyond $3.5 \mu\text{m}$, an acceptable image at $2.2 \mu\text{m}$ but hardly any image improvement at all at $1.2 \mu\text{m}$. Its Shack-Hartmann-type wavefront sensor simply ran out of photons!

Fortunately, it is possible to obtain diffraction-limited images from the ground with only about one-thousandth of the photons needed by adaptive optics. In speckle imaging, thousands of distorted exposures are taken and the clear image is reconstructed afterwards. But as the efficiency of this technique grows sharply with smaller seeing disks, so-called 'partial adaptive optics' might be combined with it. Its real-time reconstruction of the wavefront is only partial, due to the photon shortage at short wavelengths, but it would still speed up interferometric applications. Thus, ESO has not "decided to rely on adaptive optics for the VLT", as Dickman writes — this will be just one of many optical concepts that will be tried.

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Thin-film fuel cells

SIR—Dyer's (C. K. Dyer, *Nature* **343**, 547; 1990) thin-film fuel cell presents two challenges to those involved in fuel-cell research. The first is to explain his observations in terms of known electrochemistry, and the second is to evaluate the technological potential of the device relative to present 'conventional' fuel cells.

A key element of Dyer's device seems to be the electrochemical response of a given electrode to a mixture of hydrogen (or another fuel) and oxygen. For example, the voltage is reversed in sign when nickel replaces platinum as the front electrode. This is probably because at the relevant experimental conditions the rate of oxygen reduction on nickel is higher than the rate of hydrogen oxidation, whereas the converse is true for platinum.

This suggests a possible mechanism for the behaviour of a cell with two Pt electrodes exposed to H_2/O_2 mixtures. That the back Pt electrode operates as the oxygen electrode could be due to the absence of mobile anions in the electrolytes used (pseudoboehmite or Nafion) and a partial oxide coverage on this electrode (formed during fabrication). The former could enhance the rate of O_2 reduction at the Pt electrode (which is inhibited by adsorbed anions); in the latter case, a monolayer of oxide on Pt is known to inhibit H_2 oxidation very effectively, while perhaps having a smaller effect on O_2 reduction. This explanation requires no new electrochemistry.

Irrespective of the exact mechanism,

central to Dyer's device is the exposure of front and back electrodes to a mixture of fuel and oxidant, and the reliance on the relative kinetics of all possible processes at the electrode surfaces. There are potential problems with such an approach. A mixture of H_2 and O_2 in the ratios considered favourable (see Fig. 3 of ref. above) is explosive; at safer ratios, device performance would be limited by transport to the back electrode. Then there is the problem of fuel-oxidant recombination. Dyer states for one cell that fuel utilization is only 50 per cent because of recombination at the front electrode. This degree of fuel wastage is unacceptable for applications to electric vehicles. 'Conventional' fuel cells, which keep the fuel and oxidant separate, utilize nearly 100 per cent of the fuel.

Dyer emphasizes the advantage of the low operational temperatures of his device relative to high-temperature conventional fuel-cell technologies. This is somewhat misleading: low-temperature conventional fuel cells are now being developed, such as the polymer-electrolyte cell which operates best at just 80°C .

I recognize that Dyer's paper is the first description of a new idea, but I feel that progress towards desirable fuel-cell applications such as electric vehicles is more likely to come from systems with separated fuel and oxygen streams.

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A recombination

SIR—I believe the segregation advantage proposed by Kirkpatrick and Jenkins¹ cannot play a major part in the maintenance of sexual reproduction. Parthenogenesis is generally achieved by the suppression of meiosis or pre-meiotic doubling². In both cases, every daughter receives an exact copy of the mother's genome unless somatic recombination occurs.

Kirkpatrick and Jenkins ignored the effect of somatic recombination, which can greatly reduce the time a locus is heterozygously fixed in the asexual population. If the combined rate of somatic gene conversion and crossing over at the four-strand stage in mitosis is equal to α events per individual per generation, then the equilibrium number of heterozygously fixed loci in the asexual population is:

$$\bar{n} = n_0 \times \mu / (\mu + \alpha)$$

where n_0 is equation 1 in ref. 1, the equilibrium number of heterozygously fixed loci in the absence of somatic recombination, and μ is the rate of nucleotide mutation per individual per generation.

Estimates of the rate of somatic recombination vary from 10^{-3} events per gamete in diptera³ to 10^{-8} events per cell generation in yeast⁴. Studies of mosaicism in mice suggest that even in mammals somatic recombination is not uncommon⁵. Eighty per cent of somatic recombination events in yeast⁴ and mammals⁶ are gene conversions, and studies of mosaicism in diptera suggest that somatic recombination occurs at the four-strand stage⁷ of mitosis.

It therefore seems likely that the rate of somatic recombination is orders of magnitude greater than the rate of nucleotide mutation, which is about 10^{-9} events per individual per year⁸. A 100-times difference would lead to an approximately 100-fold decrease in the number of heterozygously fixed loci, and a 100th root decrease in the advantage experienced by the sexual population. The number of potential advantageous mutations required to generate a segregation advantage may become unrealistically large or impose too much load on the sexual population.

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Microtubule bundling

SIR—We recently described the bundling of microtubules into parallel arrays by microtubule-associated proteins MAP2 and tau, as well as experiments that led to a model of how this process might occur (S. A. Lewis *et al.* *Nature* **342**, 498–505; 1989). Our model depicted the formation of crossbridges between closely spaced parallel microtubules via self-interactions between a short hydrophobic α -helical region at the extreme carboxy terminus of MAP2 and tau. Our reasons for proposing the model were based on the following observations: (1) deletion of the hydrophobic α -helical domain of MAP2 eliminated the ability of the molecule to bundle microtubules without affecting microtubule binding; (2) substitution of this hydrophobic α -helical domain with the yeast GCN4 sequence (which is known to form dimers) restored the capacity to form microtubule bundles; and (3) a synthetic peptide corresponding to the hydrophobic α -helical domain of MAP2 behaved in many respects as a dimer.

Because the crosslinking of microtubules into bundles seemed to depend on the presence of a zipper-like hydrophobic domain, we decided to assay the microtubule-bundling ability of molecules

containing amino-acid substitutions that we predicted would destroy the α -helical structure of the carboxy-terminal region. Contrary to our expectations, mutations in this domain (FSZ, Fig. 1) yielded phenotypes indistinguishable from that of the wild-type molecule: all were capable of bundling microtubules to the same extent. As this experiment cast doubt on our proposed mechanism for microtubule bundling, we made 12 constructs deleting various sequences from the region of MAP2 encoding the binding/bundling domain and assayed the expressed proteins for their ability to bind to and bundle microtubules in transient transfection assays.

Although all of the new constructs led to the synthesis of properly terminated proteins, the strategem used in the construction of some of the clones (FS-H and FS-C) described in our paper led to the expression of proteins that included 29 extraneous amino acids at the carboxy terminus (zig-zag line, Fig. 1). These were encoded by a stretch of the 3' untranslated region of the MAP2 messenger RNA. Although this extraneous sequence has no effect on the results obtained with FS-C (compare FS-C with FS66, Fig. 1), it exerts a profound effect on the results

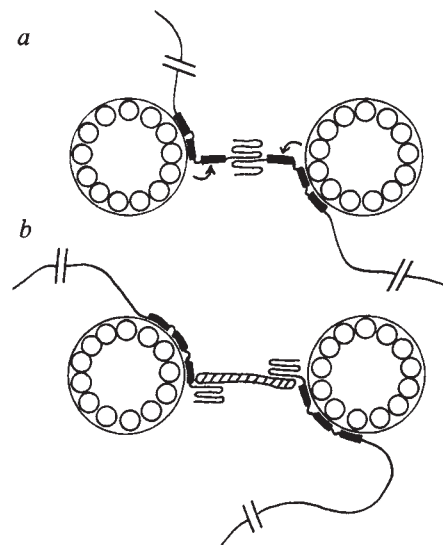


FIG. 2 Microtubule bundling by MAP2. Cross-bridged microtubules with a 20nm spacing are shown. Solid areas, imperfect repeats of the microtubule-binding domain; carboxy terminus, folded region. Hatched region in *b* is a bridging molecule unrelated to MAP2.

obtained with FS-H and leads to a complete suppression of bundling (compare FS-H with FSZ, FS10, FS11, Fig. 1). Given these new data, it is clear that the carboxy-terminal helix of MAP2 is not directly responsible for microtubule bundling as we originally proposed. The observation that substitution of this hydrophobic α -helix with the leucine zipper from GCN4 results in a construct whose expression causes microtubule bundling (FS+L, Fig. 1) must therefore be reinterpreted: the appended leucine zipper is evidently less destructive to the folding of some critical region than is the 29-amino-acid extension present in FS-H. In addition, the tendency of a synthetic peptide corresponding to the C-terminal α -helix to dimerize *in vitro* must be irrelevant to the microtubule-bundling mechanism.

Our new data indicate that amino acids 1,758–1,780 of MAP2 are involved in microtubule bundling either directly or by influencing the secondary structure of a critical region. Two ways in which these amino acids could contribute to microtubule bundling are depicted in Fig. 2. In *a*, sequences dimerize to bridge microtubules, whereas in *b* they interact with a bridging protein. In case *a* it is necessary to postulate that the third repeat is detached from the microtubule, to give a bridge consistent with the length of cross-bridges observed in electron micrographs of microtubule bundles in transfected cells. Such a mechanism could restrict bundling to regions of high microtubule density, as dissociation of a repeat would be expected to destabilize binding.

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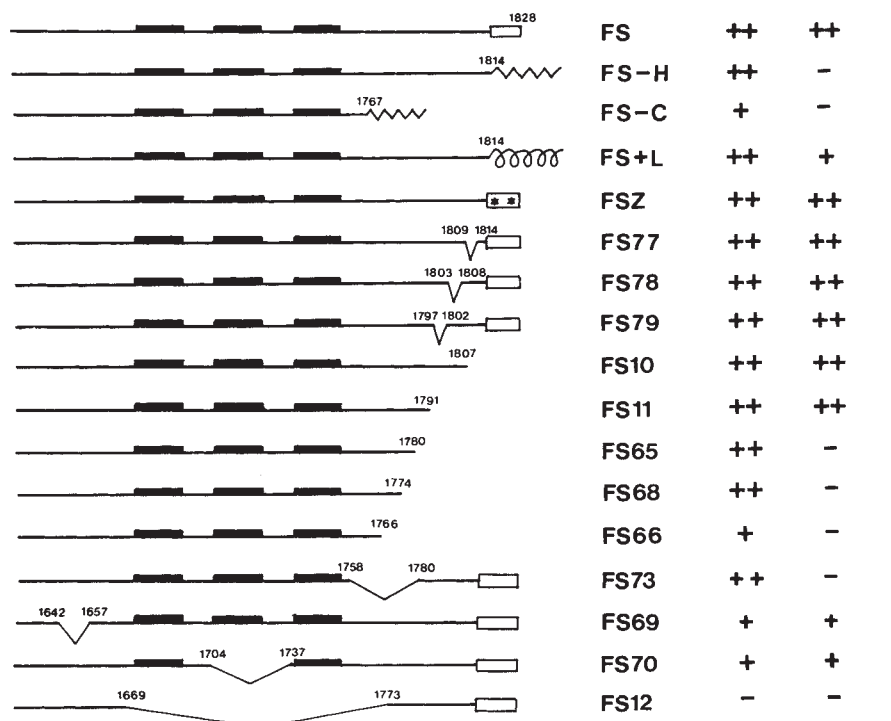


FIG. 1 Ability of MAP2 constructs to bind to and bundle microtubules. All constructs were derived from FS, a deleted version of MAP2 lacking amino-acids 228–1,621 that gives a phenotype upon transfection into cultured cells that is indistinguishable from intact MAP2. Only the region C-terminal to amino acid 1,621 is shown. Raised solid bars, three imperfect repeats forming part of the microtubule-binding domain. Open boxes, hydrophobic MAP2 carboxy-terminal α -helix. Amino-acid residue numbers at the deletion end-points of each construct are shown. Zig-zag, extraneous extension of 29 amino acids translated from constructs FS-H and FS-C; a helix that is part of the FS+L construct denotes the leucine zipper domain derived from the yeast GCN4 protein. Asterisks in FSZ, amino-acid substitutions introduced into the hydrophobic carboxy terminus to disrupt the α -helical structure. See our paper for further details.

The greatest satisfaction

Sydney Brenner

Toward the Habit of Truth: A Life in Science. By Mahlon Hoagland. Norton: 1990. Pp. 206. \$19.95, £14.95.

IN THIS autobiographical memoir, Mahlon Hoagland, one of the pioneers of the biochemistry of protein synthesis, gives a uniquely personal view of the exciting decade following on from the discovery of DNA, that very brief time in which the processes of information transfer from genes to proteins were unravelled. The author originally intended a career in surgery, but he contracted tuberculosis as a medical student, and after a long stay in a sanatorium he found that he could not withstand the rigours of medical practice and was forced to give up his internship. He came to science almost by default, and a postdoctoral fellowship brought him to the Huntingdon Research Laboratories in 1948 where he learnt how to do scientific research by working on the toxicology of beryllium. He became interested in the work of Paul Zamecnik, but between 1951 and 1953 he worked first at the Carlsberg Laboratory in Copenhagen and then in the laboratory of Fritz Lipmann at Massachusetts General Hospital before joining Zamecnik. These two years were supposed to 'retool' him in biochemistry for work on the problem of protein synthesis.

In 1941, Lipmann wrote an important review in *Advances in Enzymology* on the generation and use of phosphate bond energy. Here he introduced the notion of high-energy phosphate bonds and ushered in a new approach to theory and experiment in biochemistry which soon came to dominate the subject. All problems of biosynthesis involved two questions, one concerned with the components and the other with the energy used to put them together. Thus, in the case of protein synthesis, the components were known to be amino acids, and the central question then asked by biochemists concerned the source of energy required for making the peptide bond. By contrast, the early molecular biologists took the different view that the central question was how the amino-acid sequence was specified; the energy, they argued, would look after itself.

Armed with the methods he had learnt in Lipmann's laboratory, Hoagland discovered the mechanism of activation of amino acids soon after he joined Zamecnik's group in 1953. Earlier, Zamecnik and his associates had developed a broken cell preparation which synthesized protein, that is, incorporated added radioactive amino acids into polypeptides. They had shown that two fractions of the cell extract were neces-

sary: a sedimentable microsomal fraction and a 'soluble' fraction. The latter contained the amino-acid-activating activity. Not long afterwards, in 1955, Zamecnik discovered that the same fraction contained an RNA which could be labelled by

work on the interests of the founders, sensory neurophysiology and reproductive biology. It was here that all the basic discoveries were made that led to the development of the contraceptive pill, contemporaneously with the work of the younger Hoagland at Harvard. By the mid-1960s the Worcester Foundation was experiencing great difficulties and it is almost with a sense of relief that Mahlon Hoagland decided in 1970 to leave his unsatisfying academic post at Dartmouth to take up the leadership of the foundation. Saving the laboratory and reconstructing it have clearly given Hoagland

IMAGE
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REASONS

Mahlon Hoagland — a pioneer of protein synthesis biochemistry.

the activated amino acid. This soluble RNA — which was later called transfer RNA — was the adaptor required in Francis Crick's theory of how the DNA message might be translated into the amino-acid sequence.

Although the story of how biochemistry and genetics came to be fused into the new science of molecular biology has now been told many times, it is important to have Hoagland's account of the work of Zamecnik's group and the central importance of its discoveries, which lent reality to the ideas that flowed from the DNA structure. The discovery of messenger RNA at the end of the decade essentially completed the picture.

His work on protein synthesis and his period at Harvard form the centrepiece of Hoagland's memoir. But a second story surrounds it, and it is this that gives the book deeper interest. It concerns the Worcester Foundation for Experimental Biology, a remarkable private institution founded by Hoagland's father, Hudson Hoagland, and Gregory Pincus in 1944, to

his greatest satisfaction in life, not only for the objective value of what he created but for the personal value to him of completing the work of his father. He writes that as time passed he grew closer to his father "through our shared cultural values and our love for science and the institution we both served". And the rounded life was added to in 1980 when Zamecnik joined the Worcester Foundation after retiring from Harvard University.

We learn from a quotation of Bronowski that the habit of truth is a civilized virtue not only in science but also in art and self-knowledge. In these days, when fraud, forgery and fabrication are becoming common words in science, I suppose we need to worry about some of the priesthood discarding their habits. But there is use in these breaches in that they serve to undermine the pomposity of science and bring things down to earth by showing that scientists are just as human as politicians and businessmen. Like all human activity, science profits from the balanced conflict between the drive, passion and creativity

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of individuals and the traditions, order and discipline of our institutions. When Hoagland writes that "science is a process, a progression, because its discoveries are cumulative, the stories of an edifice under construction", we recognize the public myth that scientists are satisfied to be builders and that nobody's brick is better or bigger than anybody else's. Later in the book, Hoagland describes that when he rushed to tell Lipmann about his discovery of amino-acid activation,

Lipmann put two people to work on the same problem without even telling Hoagland. "I resented having the baton unceremoniously ripped from my sweating hand before I had even finished the first step". This is the familiar world of everyday science, and the true habit must be the truth about ourselves. □

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Sounds in concert

Christopher Longuet-Higgins

Measured Tones. The Interplay of Physics and Music. By Ian Johnston. *Hilger: 1990. Pp 397. £14.95. Distributed by AIP in the United States, \$30.*

"THE purpose of this book is easy to explain. I am a physicist and not a musician. Yet I am interested in music and am aware of just how much the two fields have to offer to one another. I would like to make this more widely known. I believe that your enjoyment of music can be increased thereby and so can your understanding of physics. Whatever your interest, a little bit of extra knowledge can only help. It is as simple as that."

Most books on science and music (the author continues) are written by physicists, who devote their earlier chapters to vibrations, waves, Fourier analysis and so on — concepts that most musical people find quite obscure. Johnston proposes to circumvent this problem by developing the physical concepts and the musical applications together. The result is a colourful patchwork of elementary physics, music theory and potted history, liberally interspersed with textbook diagrams and museum-worthy engravings. The pace of the book is evident from the synopsis of chapter 6, entitled "The Romance of Electricity": "The Romantic movement. Study of electricity, Faraday. Acoustics. Energy coupling and acoustic impedance. Mismatch theorem. Standing waves in air columns."

As Johnston warms to his task, the self-consciousness of the opening pages gives way to an engaging enthusiasm for the idiosyncrasies of musical instruments and musicians. The author keeps the pot boiling by anecdotes from the lives of the great and digressions into highbrow matters such as relativity and quantum mechanics — all conducted without recourse to algebraic mathematical equations "in the belief that they tend to frighten non-scientists". Why is it, one wonders, that supposedly educated people are so utterly

helpless in the face of a notation that is specifically designed as an aid, not a barrier, to thought? The corresponding notation for musical ideas is, of course, stave notation, and the author wisely includes an appendix on the subject. But like other writers on science and music he fails to make the point that symbols such as C, F sharp and G flat are intended to represent not so much the pitches of notes as the tonal relations between them. 'Ordinary' music has, indeed, a rich grammar, of which stave notation is the outward and visible sign, and Johnston misses a rare opportunity by not acquainting the serious reader with the unifying concept of a generative musical grammar.

Apart from the last two chapters of the volume, which provide an account of the state of the art of music technology, *Measured Tones* covers essentially the same ground as Helmholtz's immortal work *On the Sensations of Tone*, provoking the reflection that some books are serious, scholarly and systematic; others are popular, playful and picturesque. □

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New Journals

This year *Nature's* annual new journals review supplement will appear in the issue of 11 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

■ Journals which first appeared after June 1988, and which issued at least four separate numbers by the end of April 1990, will be considered for review. The deadline for submission is the end of June.

■ Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.

■ Frequency of publication must be at least three times a year.

■ The main language used must be English. Translation journals in English are eligible.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title as soon as possible to: Book Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK or 1137 National Press Building, Washington DC 20045 USA. □

Science appeal

Walter Gratzner

They're Not Dumb, They're Different. Stalking the Second Tier. By Sheila Tobias. *Research Corporation, 6840 East Broadway Boulevard, Tucson, Arizona 85710, USA: 1990. Pp.94. Distributed free.*

IT WAS, I think, an *aperçu* of Adlai Stevenson's that American society could be spared an incalculable amount of pain and expense were every child invested at birth, as part of its inalienable patrimony, with a BA or BSc degree. The balance between the two could then be adjusted each year, according to the temper of the time. Such a disposition might go some way towards meeting the issues raised in Sheila Tobias's study, which is about why so many American university students forsake science for other disciplines and how to stop them.

Tobias's title begs a question or two to start with, for it carries the merest nuance of a hint that those who prefer Greek poetry or economics or (save the mark) sociology to science might be written off by many of us as intellectually inferior (even if Tobias knows they are not); and indeed that those who are not good at science really *are* inferior and thus fit only to become hairdressers, high-court judges or merchant bankers. It also implies, does it not, that more scientists are needed and that employment appropriate to their skills awaits them? This last premise receives a passing mention near the end of the report, but is not examined.

The remit of this study then is narrow. All the same, the results make compelling reading. Tobias's touch is deft and her style refreshingly free of jargon. Her conclusions are based on the outcome of an interesting experiment: seven students (one of them a middle-aged professor of classics), who had all taken an interest in science or mathematics at an early stage in their education, were invited to take a science course and report how they got on. All are thoughtful and articulate people — several cuts, as I would judge, above the average undergraduate, and selected perhaps for that reason. Their verdicts on the merits of the several courses and on those who taught them show a striking unanimity. They found first a charged and competitive atmosphere in the classroom. Science students tended to regard their colleagues, and especially the brighter ones, as a threat; they showed none of the eager curiosity about their subjects that marked their coevals in the humanities. At the mention of any matters on which they were given to understand they would not be examined their eyes would glaze.

The presentation of the course material

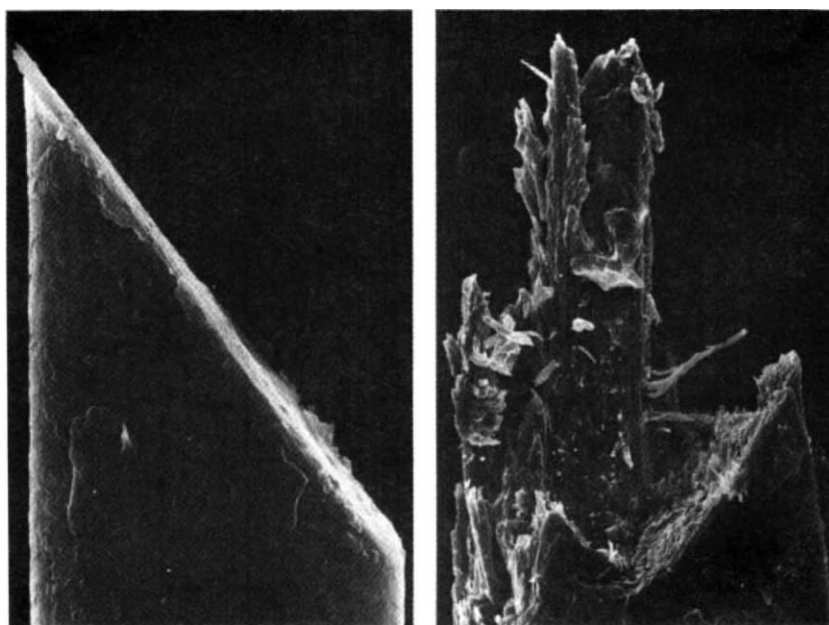
was held to be tedious, with an excessive emphasis, as Tobias's guinea pigs saw it, on "problem solving" — mastering, in other words, the techniques of the craft without reference to the purposes to which they might be put. "Too many scales, not enough music" is how Tobias sums it up. Yet this analogy brings to mind the story of how the celebrated singing teacher, Manuel Garcia, was supposed to have trained his students: for two years they were allowed to sing nothing but scales and arpeggios. Then came the moment when he would send the student on his way, with his blessing: "I have taught you all I know. Now learn some songs and operas and go into the world and make your fortune." I rather suspect that students intellectually and temperamentally drawn to science would be more likely to find satisfaction in mastering its basic grammar. One of the experimental subjects for example says of his chemistry course that more attention was paid to Avogadro's number than to Avogadro's insight. The implied plea for a more historically-minded approach to science teaching deserves to be taken seriously, but the antithesis is still a false one.

Further, the instructors were accused of having done nothing to encourage originality or audacity of thought. In short, they were failing to educate, for where they should have drawn out they sought only to bash in. Eric, one of the astutest of the seven students, gives this example:

"we were to judge the best clock for time-keeping, given a record of five clocks' readings at exactly noon. The professor chose the clock that gained exactly 51 seconds every day. I picked the clock that was within seven seconds of noon day after day. A scientist wants predictability; I would rather have convenience." Of course the professor (or the student) might also have noted that a clock that has stopped is pretty good too, for it registers the right time twice a day, whereas one that loses seven seconds a day will be exact only once in about 17 years.

When a professor was taxed with the strictures of one of Tobias's students on the quality of his chemistry course, his riposte was: "It is dull. It is dull to learn and dull to teach. Unfortunately it is the basic nuts and bolts stuff that must be mastered before anything can be accomplished. . . ." Well it is no great revelation that much (most?) university teaching is lustreless and perfunctory. Faculty members are mostly there, after all, because they want to do research; and insofar as they are willing to teach, they will invariably prefer, as at least one of Tobias's subjects also perceives, to occupy themselves with professors of the future (who, when their time comes, will aim to create yet more professors). But there is alas no seller's market in professors — and besides, embryo professors are the students least in need of teaching.

Tobias's investigation was spurred by



The scanning electron micrograph on the left is a man's beard hair, cleanly sliced by the blade of a 'wet' razor. On the right is a beard hair from the same man, torn by the action of an electric razor (both micrographs $\times 195$). These pictures from the new paperback edition of *Under the Microscope: A Hidden World Revealed* by J. Burgess, M. Marten and R. Taylor, form part of an intriguing array of pictures representing the everyday world seen under close scrutiny. The human body, seed plants, inorganic world and industrial world are some of the areas that are explored. Informative captions accompany each picture and the volume ends with a useful technical appendix. Published by Cambridge University Press on 12 July, £12.95, \$19.95.

the reported drop of 50 per cent in the proportion of US students choosing to graduate in science between 1966 and 1988. Yet whether this has any more bearing on the progress of the Great Society than has the "functional illiteracy" of 25 per cent of American youth, or indeed ex-president Reagan's arresting opinion that trees cause pollution, cannot be determined. Such (not wholly disinterested) bodies as the AAAS and the NSF have, it is true, asserted otherwise, and foresee a desperate dearth of science graduates in the next decade; but perhaps the drift from science simply reflects the level of opportunity, not to mention emolument,

available to young scientists today. In Britain such must undoubtedly be the case. In the United States, students do at any rate have the chance of dropping out of science, having at least been exposed to a little of it, and into the humanities. In Britain, if they get to university at all, a change of subject is seldom a realistic option. If British students choose to throw in the diaper and seek their future in accountancy, it may after all then be not because they are dumb but because they are smart. □

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Starting matter

David W. Hughes

The Origin of Comets. By M. E. Bailey, S. V. M. Clube and W. M. Napier. Pergamon: 1990. Pp. 577. Hbk £39, pbk £19.59.

PEOPLE studying the origin of comets are confronted with an extremely difficult task. Comets are not like stars — we cannot see regions of space in which comet formation is taking place at the present time. The strong possibility that cometary formation took place 4,570 million years ago, at the dawn of the Solar System, helps little too. Comets are not like asteroids — they do not fall to Earth — so our knowledge of their composition is rudimentary. We know that the cometary nucleus is made up of snow and dust, and that most of the snow is H₂O and the dust is similar to primitive carbonaceous chondritic meteorites. But we have little idea as to the minor constituents, or whether the composition is the same

throughout the nucleus, or whether all comets have the same composition, or the exact nature of the size distribution of the dust. Astronomers, so far, have recorded about 700 comets out of a suggested population of around a million million in the Solar System. And these 700 represent a very biased selection. The vast majority of the comets known to us happen to be the ones that get to within the orbit of Mars, and even so, only the large ones of that group have been detected.

Only one comet has been visited by spacecraft, and even here, the 16 × 8 × 8-kilometre nucleus was imaged at a resolution of around 200 metres from a closest approach distance of 596 kilometres, while the craft was travelling past at 150,000 miles per hour. So we have a crude idea as to what the nucleus of comet Halley looks like today, but because of its many close perihelion passages, the nucleus has decayed to half its original size, and so we have no idea as to its birthday appearance. Furthermore, comet Halley has an intermediate orbital period, is retrograde and is very much a

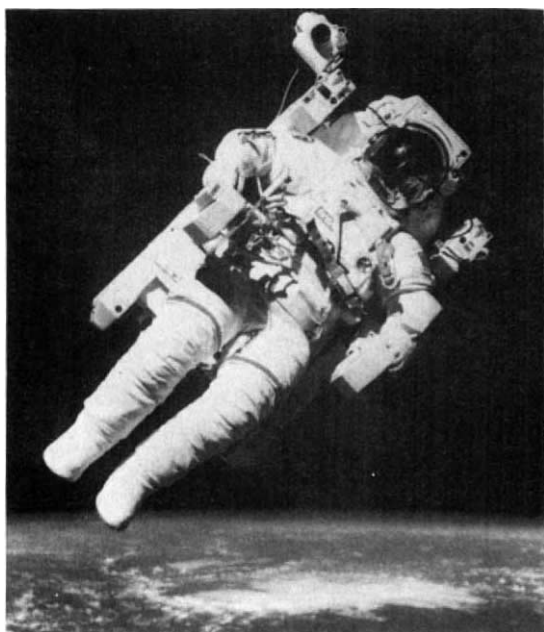
giant, so it could be far from typical. The nucleus has a density that has been estimated to be anywhere between 1.2 and 0.2 grams per cm³. So the relationship between the cometary mass and any observable brightness parameter is rather vague, and the suggestion that the cometary mass distribution is the product of accretion as opposed to fragmentation is subject to debate. The present cometary population has a total mass of the order of that of the Earth, this being a mere shadow of the initial total mass.

Cometary evolution has been very wasteful. Many comets have decayed completely into streams of meteoroid dust, and many have been ejected from the Solar System to wander off around the galactic disk. All stars with planets are thought to have produced comets in profusion, yet we have recorded no obvious hyperbolic comets passing the Sun — we see only the well-mixed remnants of our own cometary cloud and none from other systems. And seeing only a remnant, it is difficult to judge whether this fragment is typical of the original group or whether it had some rather unusual initial orbital characteristics that helped it survive. The subject is also enlivened by the probable existence of an Oort–Öpik belt of comets in a deep-freeze store of radius about a third of the distance to the Sun's stellar neighbours, and a Kuiper cloud of comets in an undetectable refrigerator just beyond the orbit of Pluto.

Bailey, Clube and Napier here provide a magnum opus that seemingly leaves no stone unturned. The reader is treated to an intensive review of mankind's historical notions as to the structure and significance of comets. Much is made of the present-day fashionability of catastrophism, and giant comets, cometary fragmentation and times of high impact flux are hinted at as having occurred in the not too distant past. Detailed analyses are presented of work done in the field of orbital perturbation. The efficacy of Jupiter as the capturer of passing long-period comets is debated in depth. The stability of the Sun's surrounding Oort–Öpik cloud of comets, against the passage of stars and the general galactic tides, that varies in strength as we wobble our way around the galactic nucleus, is investigated.

The Origin of Comets is an authoritative, well-referenced, well-illustrated, comprehensive and profound review of an extremely complex but fascinating minor topic in cosmogony. The fact that we are still not sure where comets came from, or might still come from, does not stop this book being most enjoyable and rewarding. I found it continuously thought-provoking and a spur to further endeavour. □

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The world's first human satellite — Bruce McCandless, 7 February 1984, flies the manned manoeuvring unit. *The Illustrated Encyclopedia of Space Technology* (revised edition) by K. Gatland, from which the photograph is reproduced, is a colourfully illustrated volume that documents the story of rocketry, examines military space systems, follows launch vehicle development, the use of satellites, the Challenger disaster and the Voyager encounters of Uranus and Neptune. Published by Salamander, distributed in the UK by Hodder and Stoughton £16.95; in the US by Orion \$29.95.

The fuelling of active galactic nuclei

Isaac Shlosman, Mitchell C. Begelman & Julian Frank

We review accretion mechanisms for powering the central engines of active galactic nuclei (AGN) and possible sources of fuel. Local sources, such as dense star clusters, require extreme set of parameters. Instead we argue that the interstellar matter in the main body of the host galaxy is channelled towards the centre and address the problem of angular momentum transport. Thin accretion disks are not a viable means of delivering fuel to luminous AGN on scales much larger than a parsec because of the long inflow time and effects of self-gravity. There are also serious obstacles to maintaining and regulating geometrically thick, hot accretion flows. We emphasize the role of non-axisymmetric perturbations of the gravitational potential on galactic scales and their triggers: galaxy interactions and internal self-gravitational instabilities. We outline a unified model for fuelling AGN, in which the inflow on large scales is driven by gravitational torques, and on small scales forms a mildly self-gravitating disk of clouds with inflow driven by magnetic torques or cloud–cloud collisions.

THE term ‘active galactic nucleus’ refers to nuclei that exhibit an anomalous energy output, compared to that expected from a ‘normal’ mixture of stars and gas. It is usually reserved for activity that appears to be non-stellar in origin and confined to the inner few parsecs of a galaxy, although it is sometimes used for ‘starburst’ galaxies (SBG), in which the energy comes from a high formation rate of massive stars in the inner few kiloparsecs¹. We concentrate on non-stellar activity, but point out how some of the fuelling mechanisms may also trigger SBGs. A wide range of phenomena is associated with non-stellar activity, including: broadband spectra, from radio frequencies to γ -rays; rapid variability, indicating an extremely compact source; luminosities that may exceed the stellar luminosity of the host galaxy; strong broad emission lines, apparently due to photoionization; and relativistic jets, observed to emanate from regions less than a parsec across. Recent reviews deal with observations and classification schemes for AGN^{2,3}. It is generally accepted that this activity results from the interaction of matter with a supermassive black hole (SBH) of mass $\sim 10^3$ – $10^{10} M_\odot$ (ref. 4).

In SBH models for AGN, the required accretion rate is given by $\dot{M} \approx 0.2(\epsilon/0.1)^{-1} L_{45} M_\odot \text{ yr}^{-1}$, where L_{45} is the energy output in units of $10^{45} \text{ erg s}^{-1}$ and ϵ is the mass-to-energy conversion efficiency. The problem of ‘fuelling’ an AGN has two parts: what is the source of the fuel, and how is it channelled into a region that is tiny by galactic standards—comparable in scale to the Solar System? The most luminous AGN (quasi-stellar objects [QSOs] and giant radio galaxies) have $L \geq 10^{47} \text{ erg s}^{-1}$, requiring rates of 10 – $100 M_\odot \text{ yr}^{-1}$; finding enough fuel to power these objects is a challenge. For the majority of AGN, which are much less luminous, the main problem is to get the gas to flow into the centre, which means losing nearly all the angular momentum of the gas. AGN models without an SBH, such as ‘warmers’ still require large amounts of material to be channelled towards the centre.

We first discuss models that have a ‘local’ source, usually a dense star cluster with a core radius of a few parsecs; in these, it is difficult to supply mass at a rate adequate to fuel the most luminous AGN. Furthermore, clusters of the requisite density and compactness have never been observed, and no compelling theoretical arguments demand their existence.

We therefore review at length those models in which the mass supply originates on scales of a kiloparsec or more, either from the interstellar medium (ISM) of the host galaxy or from outside the galaxy through infall of intergalactic gas or galaxy interactions. We show why inflow from a thin accretion disk is not effective beyond a few parsecs from the SBH, and consider possible alternatives, including ‘hot’ accretion flows, thick disk-like structures consisting of clouds, and magnetic torques. On

the largest scales (beyond a few tens of parsecs) inflow is likely to be driven by gravitational torques. We discuss how each of these mechanisms contributes to the fuelling, and how an episode of activity may be triggered. We take issue with the view that the trigger for nuclear activity is usually external to the galaxy, that is, a tidal encounter or merger with another galaxy. We argue that such interactions comprise but one among a number of possible triggers, and that activity may be induced in the course of ‘normal’ galactic evolution.

Finally, we list key observations, becoming feasible with next generation of instruments, that could shed light on these problems.

Dense star clusters

Models in which a dense star cluster sits at the centre of an active galaxy assume that $\sim 10^9$ stars have been placed at $r \leq 10 \text{ pc}$ by stellar and/or gas dynamical processes. The challenge to any of these models is to account for the formation of the central mass concentration because once the mass has been brought to $r \leq 10 \text{ pc}$, the angular momentum problem has largely been solved.

A time-averaged gas production rate by Population II (aged stars) is $\sim 0.01 M_\odot \text{ yr}^{-1}$ per $10^9 M_\odot$ of stellar mass. For a coeval population of main-sequence stars the gas liberation rates are higher at early times. Apart from the fact that the gas liberated by stars in the central cluster is insufficient to power the brightest sources, the ultimate fate of this gas is uncertain because of the heating by supernovae. This has led some authors to consider unusual populations and stellar evolution in a strong radiation field.

Gas release through tidal disruption (TD) of stars^{6–8} suffers from serious drawbacks: the disruption rate is not sufficient to power QSOs unless physical collisions dominate^{9,10}; and disruptions cease when the mass of the SBH exceeds a critical value $\sim 10^8$ – $10^9 M_\odot$ (above this mass, stars are swallowed whole without emission of radiation^{6,11}). The presence of a central gas cloud and tidal heating may enhance the rate of captures. For tidal disruptions to occur in a quasi-steady state, stellar orbits must diffuse by two-body relaxation towards lower angular momentum orbits until they enter a small ‘loss-cone’ of semi-aperture $\theta_{\text{crit}} \approx (t_{\text{dyn}}/t_R)^{1/2}$ (refs 7, 8). The maximum attainable fuelling rate for spherical clusters with near-isotropic velocity distributions is of the order of the core mass per relaxation time^{9–11}

$$\dot{M}_{\text{TD}} \approx \frac{N_c m_*}{t_R \ln(2/\theta_{\text{crit}})} \approx \frac{m_*}{t_{\text{dyn}}} \quad (1)$$

The relaxation time t_R is $\sim N_c/\ln N_c$ times longer than the

dynamical time t_{dyn} , where N_c is the number of stars in the cluster and the typical stellar mass is m_* . This limit can be exceeded by an order of magnitude if the potential is triaxial^{12,13}. The disruption rate is, however, still below the level required to power luminous QSOs. Closely related ideas are the scattering of stars into loss-cone orbits during a merger and formation of a binary black hole^{14,15}. If tidal disruptions occur in nearby galaxies and weaker AGN, they may have interesting observable consequences; recent studies have dealt with the distribution of binding energies in the debris^{16,17} and the viscous evolution of the debris after circularization¹⁸.

Early studies estimated the amounts of gas liberated in disruptive collisions and dealt with the possibilities of stellar mergers, expected to occur at low velocities, and of 'traversals', expected when a dense star impinges on a tenuous giant. The most recent simulations for equal-mass stars re-evaluate the fraction of escaping gas and include grazing collisions¹⁹. If all the debris of stellar collisions are accreted by the black hole then it is possible to obtain the required fuelling rates^{9,10}. Collisional disruption occurs when the typical stellar relative velocity v_c exceeds the escape velocity from the star v_* . Thus the maximum rate of gas production by collisions is given by

$$\dot{M}_{\text{coll}} \approx \frac{N_c}{t_{\text{coll}}} \approx \frac{m_*}{t_{\text{dyn}}} \left(\frac{v_c}{v_*} \right)^4 \approx \dot{M}_{\text{TD}} \left(\frac{v_c}{v_*} \right)^4 \quad (2)$$

Most of the debris are released at high velocities ($\sim v_c$) and could in principle escape accretion¹⁹.

The cluster model has recently experienced a revival by investigators adopting an *ad hoc* population of stars, or considering the effects of ablation and radiation heating to overcome the fundamental problems. The intense radiation field of the central engine may lead stars to expand, and increase the mass-loss rate by a combination of effects: stronger winds, more weakly bound outer layers, and increased rate of collisions and tidal disruptions^{20–23}. The rate of ablation by the interstellar medium (ISM) is limited by the momentum flux incident upon the star²², $\dot{M}_{\text{abl}} \approx \rho v_c \pi R_*^2 \approx (\rho/\rho_c) \dot{M}_{\text{coll}}$, where ρ is the density of the ISM, R_* is the radius of the ablated star, and ρ_c is the distributed mass density of the cluster. Thus, ablation is important only when direct gas dynamical processes in the ISM are dominant.

A related suggestion is that 'bloated' stellar atmospheres ionized by ultraviolet and X-rays from the central source may be identified with the broad-emission-line clouds^{20,24,25}. Recent one-dimensional simulations of the evolution of irradiated stars suggest that not much can be gained from the 'bloating' of a star. Although red giants may lose their envelopes faster, a population of coeval stars may live longer on the main sequence and the total mass loss may be reduced if the active phase lasts less than $\sim 10^9$ yr (ref. 26).

The star clusters required for powerful AGN cannot be the result of evolution by two-body relaxation as collisions would destroy the cluster when v_c is driven significantly above v_* . It is conceivable that gas dynamical processes such as a sequence of bar instabilities²⁷, in which the gas aggregates into a bar, or galaxy collisions²¹ might trigger star formation in the nucleus, resulting in very dense clusters. These clusters might evolve into a moderately massive black hole through a gas dynamical phase²⁸ or by a relativistic instability²⁹, although the onset of the latter is questionable even at large central redshifts³⁰. Cluster evolution models still fail to produce luminosities much in excess of 10^{45} erg s⁻¹, despite the assumption of an initial phase where gas is stored in a 'reservoir' (ref. 31).

To summarize, the main drawback of cluster models is that one must assume at the outset the existence of a dense and massive cluster. To fuel luminous AGN, the clusters must be far more compact than any observed so far in the nuclei of galaxies. Because the cluster is formed from material that presumably started with a much larger specific angular momentum, existing models beg the question of angular momentum transport.

Thin accretion disks?

Outside the galactic nucleus lies a vast reservoir of fuel—the ISM of the host galaxy. Its distribution and amount vary widely, but generally there seems to be enough gas to fuel a prolonged episode of activity, provided that the material can lose angular momentum in a sufficiently short time. The thin accretion disk³² seems to be the main mechanism for angular momentum transport in mass-transferring binaries, protostars, and possibly in the innermost regions of AGN. But large-scale fuelling probably cannot be achieved by means of a scaled-up version of such a disk.

First, the viscous inflow time, $t_{\text{visc}} \approx 10^9 \alpha^{-1} T_{100}^{-1} v_{\phi,100} r_{10}$ yr, tends to be too long at radii larger than a few parsecs. Here α is the ratio of shear-induced stress to pressure in the disk (usually assumed to be ≤ 1), the disk temperature T_{100} is in units of 100 K, the orbital speed $v_{\phi,100}$ is in units of 100 km s⁻¹, and r_{10} is the radius in units of 10 pc. If t_{visc} is longer than the lifetime of the AGN (let alone the Hubble time) then the disk cannot fuel the AGN steadily from large r .

Second, thin gaseous disks are locally Jeans unstable when the mean density becomes so large that the self-gravity exceeds the tidal gravitational field³³, that is, when $n \geq n_{\text{crit}} \approx 10^6 v_{\phi,100}^2 r_{10}^{-2}$ cm⁻³. This leads to a critical accretion rate, $\dot{M}_{\text{crit}} \approx 3 \alpha c_s^3 / G \approx 5 \times 10^{-4} \alpha T_{100}^{3/2} M_{\odot} \text{ yr}^{-1}$, above which the disk is locally unstable to clumping^{34,35}. Here c_s is the thermal sound speed or velocity dispersion in a turbulent disk. We note that $\dot{M}_{\text{crit}}$ is independent of both r and v_{ϕ} . Thus thin accretion disks cannot carry enough mass to fuel luminous AGN, nor transport it inward on a short enough timescale, unless $\alpha \gg 1$ and/or $T > 10^4$ K. Can either of these conditions be met?

If the disk were optically thick in the far-infrared and heated only by viscous dissipation, its temperature would be $T_{\text{eff}} \approx 11 (\dot{M}/1 M_{\odot} \text{ yr}^{-1})^{1/4} v_{\phi,100}^{1/2} r_{10}^{-1/2}$ K. The actual temperature will be higher, partly because of the effect of finite optical depth and partly because local dissipation is unlikely to be the main heating mechanism in the disk at the radii we are considering. Other sources of heating may include radiation directly incident on the disk from the central engine^{36,37}, radiation scattered down onto the disk^{34,35,38}, radiation emitted by jets³⁹, and starlight. Whereas the surface flux owing to viscous dissipation decreases with radius as r^{-3} , the incident flux, if present, is likely to decrease less rapidly. With an incident flux of ~ 1 –10% of the AGN luminosity, the temperature in the dust-free disk drops below $\sim 10^3$ K at $r \geq 1$ pc. When dust is present at normal ISM abundance, the thermal properties of the disk are completely dominated by the radiative heating and cooling of dust, and the temperature will drop below 100 K at ≥ 10 pc even in luminous AGN.

Radiation therefore cannot keep a thin disk warm enough to carry the required mass flux without becoming gravitationally unstable, unless $\alpha \gg 1$. If the disk can be kept in a highly turbulent state, larger mass flow rates are possible. There are at least two effects that might maintain significant level of turbulence, or lead to an effective $\alpha \gg 1$: local gravitational instabilities and energy input associated with star formation.

Local gravitational instability will lead to the growth of density and pressure inhomogeneities with increasing peculiar velocities. If these inhomogeneities (clumps) interact with one another and with the mean flow, they transport angular momentum. Provided that the clumps are prevented from collapsing, the α thus obtained ranges from $\ll 1$, if the disk is kept at marginal instability⁴⁰, to $\gg 1$, if the disk is strongly unstable⁴¹. Alternatively, if the self-gravitating clumps contract to the point where they hardly interact at all, the disk will resemble more a stellar disk (or a disk of individual clouds) than a turbulent disk³⁵. In this case, we would expect the effective α to decrease with the development of instability. To prevent the disk from fragmenting into weakly interacting clumps, the cooling time of the gas t_{cool} , must be longer than the time required for contraction (gravitational free-fall time in the clump). This condition

imposes an upper bound on α of a few (ref. 42). It therefore seems more likely that a self-gravitating thin disk would fragment rather than develop α large enough to feed a luminous AGN.

The likelihood of fragmentation suggests that we should examine the consequences of star formation in a self-gravitating disk. Specifically, will heating by stellar winds and supernovae lead to a higher temperature and/or greater disk turbulence, resulting in faster inflow? The cumulative effects of star formation depend on its rate or 'efficiency' ξ , defined as the fraction of mass going into stars per local free-fall time. The timescale for turning the disk into stars is $\sim 4 \times 10^6 (\xi/0.05)^{-1} (n/n_{\text{crit}})^{-1/2} v_{\phi,100}^{-1} r_{10} \text{ yr}$, which is much shorter than the inflow time t_{visc} . A self-gravitating disk is therefore expected to be significantly depleted by star formation.

The total rate of energy input from T Tauri stars and radiation-driven winds and supernovae, $\dot{E}_* \approx 10^{41} (\xi/10^{-3}) (n/n_{\text{crit}}) T_{100}^{1/2} v_{\phi,100}^2 \text{ erg s}^{-1}$, is marginally enough to keep the disk warm and/or turbulent. The efficiency is now normalized by 10^{-3} because the high-mass stars dominate the energetics (by a factor of ~ 100), even if the initial mass function is heavily weighted towards low-mass stars. Only a small fraction ($\sim 1\%$) of this energy is actually available for heating the disk. The rest goes into radiation through the cooling of shocked gas, or is lost through the 'breakout' of blast waves or wind-driven bubbles from the disk^{35,43}.

Star formation in a locally self-gravitating disk will leave behind a disk of stars, which should persist to the present time because two-body relaxation operates slowly. Observations suggesting the presence of a SBH in the nuclei of nearby galaxies display a high degree of flattening in the circumnuclear regions and indicate the importance of rotation⁴⁴⁻⁴⁶. We conjecture that these systems could have been formed by the fragmentation of self-gravitating disks.

A 'hot' accretion flow?

There are good reasons to consider an accretion flow that starts out hot ($\gg 10^4 \text{ K}$). First, it is well known that accretion disks

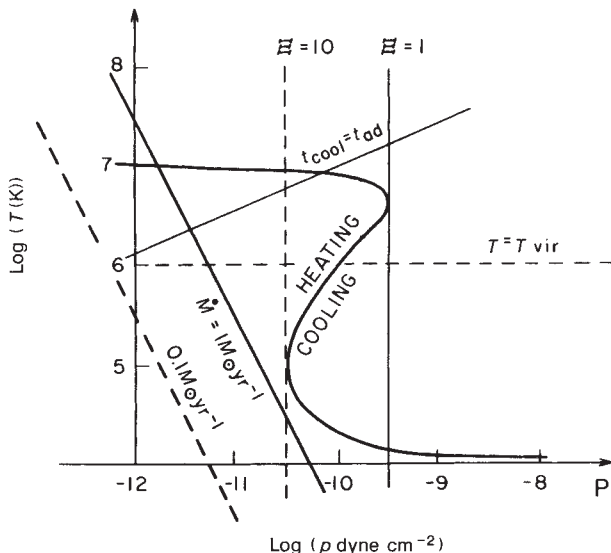


FIG. 1 Curves of constant $\dot{M}=1$ and $0.1 M_{\odot} \text{ yr}^{-1}$ on the p - T plane for $\alpha = v_{\phi,100} = r_{\text{kpc}} = 1$. The horizontal line labelled ' $T = T_{\text{vir}}$ ' indicates the (virial) temperature above which the gas will tend to escape from the gravitational potential, rather than accrete. Also plotted is the curve ' $t_{\text{cool}} = t_{\text{ad}}$ ', below which radiative cooling dominates over the compressional (and dissipative) heating associated with the accretion flow. The threshold for runaway heating is shown schematically, assuming an ionizing luminosity of $10^{45} \text{ erg s}^{-1}$ and an AGN spectrum with an inverse Compton temperature of 10^7 K . High-accretion-rate flows needed to power luminous AGN ($\geq 10 M_{\odot} \text{ yr}^{-1}$) require pressures well in excess of $10^{-10} \text{ dyne cm}^{-2}$ for $T < T_{\text{vir}}$. Under these conditions only external source of heating can prevent the flow from catastrophic cooling and collapsing into a thin disk.

can exhibit a thermal bistability^{47,48}. Second, there is strong observational evidence that cooling flows carry large quantities of hot gas into the central regions of many early-type galaxies, some of which contain AGN^{49,50}. Third, hot flows can support much faster inflow with the same α , and can carry more mass without becoming gravitationally unstable.

We first consider hot flows that have significant rotational support, are in vertical hydrostatic equilibrium, and rely on momentum transport with some characteristic $\alpha (\leq 1)$. Normalizing the pressure to $10^{-12} \text{ dyne cm}^{-2}$ (a few times the pressure in the ISM of the Milky Way), the temperature to 10^6 K and the radius to 1 kpc , the accretion rate is $\dot{M} \approx 0.2 \alpha p_{-12} T_6^{1/2} v_{\phi,100}^{-2} r_{\text{kpc}}^2 M_{\odot} \text{ yr}^{-1}$. Figure 1 shows curves of constant $\dot{M}$ on the p - T plane. Pressures two to three orders of magnitude above typical ISM pressure are required to obtain the fuelling rate for a luminous AGN without exceeding the virial temperature T_{vir} . Such pressures are inferred to exist in many early-type galaxies⁵¹. But some external source of heating is required to prevent the flow from cooling and collapsing into a thin disk (see Fig. 1). The requirement of extra heating is even more pronounced at smaller r .

A high heating efficiency by supernovae and winds from massive stars requires that the energy be distributed throughout the hot gas before localized regions have a chance to cool. Unless there is some feedback regulating the formation rate of massive stars, this mechanism seems to be unstable: if T decreases, so does the heating and the gas will collapse onto the central plane; if T increases, the cooling becomes less efficient and the gas will begin to flow out in a wind. The outflowing gas might not escape the galaxy completely, particularly if a massive halo is present. Instead, adiabatic cooling of the outflowing gas could lead to the condensation of clouds, which fall back into the galaxy and ultimately join the accretion flow (as in 'galactic fountain' models for the ISM⁵²).

A second possibility is that the gas is heated by X-rays from the central source. If the ionization parameter Ξ (ionizing radiation pressure/gas pressure) > 1 , then the gas will begin to heat towards the inverse Compton temperature of the radiation field, which probably exceeds T_{vir} in most cases. This would occur in less than the inflow time⁵³. Thus, this mechanism also tends to produce a wind, rather than to promote steady accretion.

If angular momentum transport is more efficient (owing to organized magnetic fields, for example), or if the gas is prevented from blowing away by the pressure of the intergalactic medium, then hot flows may be a rather efficient way of fuelling AGN. Cooling-flow models with inflow rates of $\sim 1 M_{\odot} \text{ yr}^{-1}$ have been constructed to explain the X-ray observations of normal elliptical galaxies⁵⁴. Adequate inflow rates of hot gas may reach the nucleus even if matter continuously drops out of the flow owing to thermal instability^{55,56}. Finally, a hot galactic atmosphere may provide a means of extracting angular momentum from a cold disk embedded in it, through some sort of frictional or hydrodynamic coupling⁵⁷.

A disk of clouds

Clearly, idealized models of quasi-continuous accretion flows run into serious difficulties. In a straightforward analogy with the theory of Saturn's rings⁵⁸, we describe an alternative type of flow in which the 'disk' is composed of randomly moving clouds having a small volume filling factor, which are embedded in a low-density medium⁴². The viscosity is provided by collisions between clouds. The results can be summarized in terms of the cloud covering factor C and effective α

$$\alpha \approx \begin{cases} C & C \ll 1 \\ C^{-1} & C \gg 1 \end{cases} \quad (3)$$

Transport of angular momentum through cloud collisions entails viscous dissipation, which increases the cloud velocity dispersion Δv at the expense of the free energy associated with shear,

provided that the collisions are sufficiently elastic. This would probably require clouds in which the gas pressure is strongly dominated by magnetic energy⁵⁹. Stellar winds and supernovae do not seem to be promising sources of energy because they are used inefficiently. A third possible source of energy is associated with local self-gravity in the disk. A disk of clouds with $C \ll 1$ will be subject to stellar dynamical analogues of the local instabilities that afflict self-gravitating gaseous disks. The ultimate outcome of these instabilities is to thicken the disk until $\bar{n} \approx n_{\text{crit}}$. If $C < 1$, the cloud collision time is longer than the growth time of instability and the disk can be kept at $\bar{n} \approx n_{\text{crit}}$ through continuous mild instability. The resulting accretion rate

$$\dot{M} \approx \frac{3\alpha(\Delta v)^3}{G} \approx 700 C \left(\frac{\Delta v}{v_\phi}\right)^3 v_{\phi,100}^3 M_\odot \text{ yr}^{-1} \quad (4)$$

is sensitive to the highly uncertain value of Δv , which depends on the properties of the clouds and their collisions.

If the magnetic field is dynamically important, the magnetic energy will compete with the kinetic energy of cloud random motions. Clouds trapped on magnetic loops could exhibit large dispersion velocities with a low frequency of collisions, affecting the transport coefficients. Predicting the outcome is beyond the scope of this discussion.

Although the ISM in the Milky Way and other nearby spirals has a cloudy structure, the inflow rate due to cloud-cloud collisions is too small to fuel luminous AGN. At large distances this is primarily because the velocity dispersion is too small. In

the molecular torus at the Galactic Centre the velocity dispersion and covering factor of clouds are moderately high, but $\bar{n}$ is well below n_{crit} (ref. 60).

Large-scale non-axisymmetric accretion: triggers

Large-scale gravitational^{61,62} and hydromagnetic^{63,64} torques are non-local mechanisms for coupling fluid elements, in contrast to the local viscosity. Angular momentum transport by non-local mechanisms avoids the constraints that limit the effective viscosity (such as the sonic limit for turbulence) in local mechanisms.

Magnetic stresses can certainly deal with the excess angular momentum in strongly magnetized accreting objects and certain types of close binaries. Ordered magnetic fields on scales of ~ 10 pc in the form of loops and arcs have been identified in the Galactic Centre⁶⁵ and could, if dynamically important, drive accretion in the inner few tens of parsecs from the SBH⁶⁶. On larger scales, however, gravitational torques will dominate. It is for this reason primarily that we concentrate on the latter.

Mechanisms that excite large-scale non-axisymmetric modes in a stellar galactic disk can be classed as extrinsic or intrinsic (Fig. 2). The former include tidal encounters between galaxies (fly-bys) and orbital torques by satellites (with mergers as an advanced stage). The latter include instabilities brought about by the evolution of the disk (such as infall or dissipation) and distortions built in by initial conditions (such as a triaxial potential). In the absence of a comprehensive theory of self-gravitating disks, empirical methods have been used to map out domains of stability. *N*-body simulations of stellar disks have revealed a strong tendency for low-order modes, particularly $m = 2$ (bar-like) modes, to grow in the absence of inner Lindblad

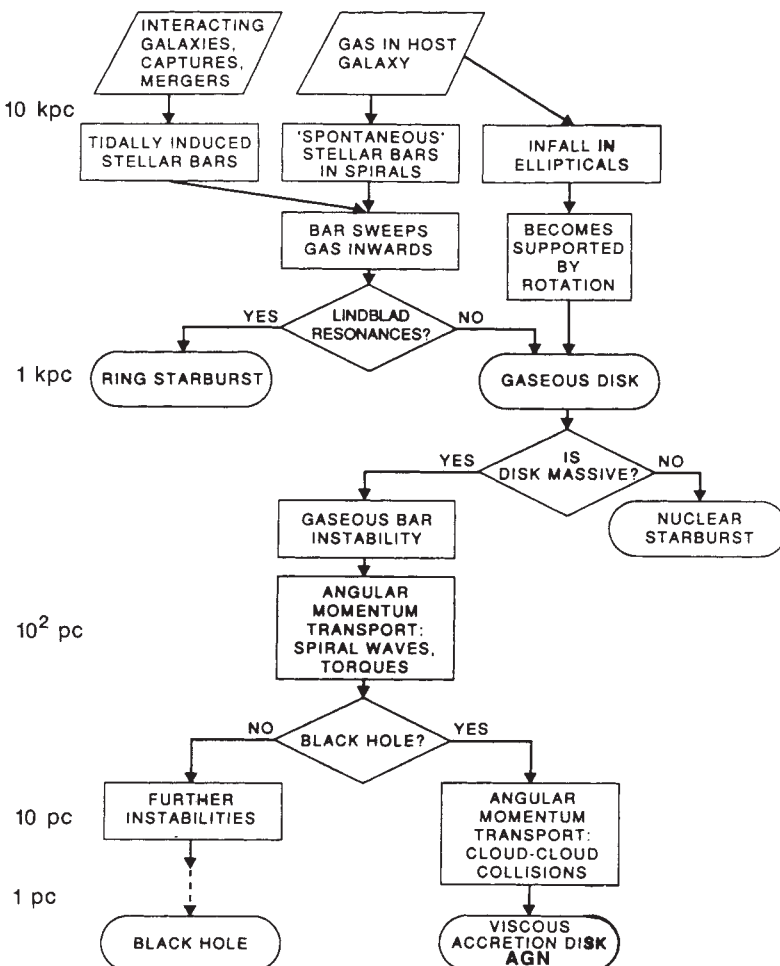


FIG. 2 Unified model for fuelling AGN: different pathways by which host galaxies can acquire a massive black hole and/or become an AGN or a SBG. The approximate radial scales at which these processes take place are indicated on the left.

resonances⁷² (ILRs) and a hot massive halo. Numerical modelling^{67–70} has shown that tidal interactions between galaxies, both fly-bys and mergers, usually lead to transient stellar bars. Furthermore, a tidal interaction strengthens an already existing bar, provided that the collision is not head-on⁷¹. But a bar may also be a long-lived and self-sustained configuration⁷² resulting from a global self-gravitational instability⁷³, or may be a relic from the galaxy formation epoch. What makes the stellar bar-like mode attractive as a mechanism for fuelling nuclear activity is the almost universal response it triggers in the gas: a pair of large-scale shocks. In contrast, if the stellar disk of an interacting galaxy is prevented (artificially) from being deformed, the gaseous component responds only weakly to a direct tidal perturbation.

Gas is a sensitive tracer of the underlying gravitational field: weak deviations from axial symmetry produce large variations in the gas density and velocity distribution owing to cloud–cloud collisions. The dissipational gaseous component is important: a collisionless system close to dynamical equilibrium will show almost no infall, even when torques are applied. Here we shall not distinguish between bars and oval distortions of stellar galactic disks—both have similar effects on the gaseous component⁷⁴. A bar-like deformation typically has a radial wavelength comparable to the size of the system and therefore provides a long-distance coupling between fluid elements, ensuring efficient outward transport of angular momentum on the dynamical timescale. Inside the co-rotation radius of barred spirals, the gas is shocked on the leading edge of the bar and, in the absence of ILRs, flows inwards on a dynamical timescale, accumulating in the circumnuclear region of ≤ 1 kpc (refs 27, 74–76). Further inflow is slow because a large-scale stellar bar potential with a radial extent of several kpc becomes weak at $r \leq 0.1 R_{\text{bar}}$. If ILRs are present, a ring-like gas distribution may result at the position of the resonances (where the torques applied to the gas vanish). In most numerical simulations of stellar bars, they form as short, rapidly rotating structures with no ILRs, and then slow down and grow toward the co-rotation radius as a result of angular momentum transfer to the outer disk⁷².

The idea that the tidal effects of companion galaxies trigger inward mass motions is an old one^{61,77}. Galaxies that are obviously interacting do show stronger than average H α or far-infrared luminosities^{78–80}, although morphological peculiarities *per se* do not seem to be a predictor of enhanced emission characteristics^{81,82}. In some cases massive stars seem to be forming at an exceedingly high rate ($\sim 100 M_{\odot} \text{ yr}^{-1}$) in a circumnuclear ring of molecular gas ($r \approx \text{few kpc}$) or in the innermost parts of the disk ($r \leq 1$ kpc). Whether fly-bys have a direct effect on the inner parsec and cause an increased mass supply to the SBH is controversial. Early results by Dahari⁸³ indicated that Seyferts are about five times more likely to have close companions than are field galaxies, but Dahari's control sample did not match the Seyfert sample in properties other than nuclear activity. Hence it is not clear whether the excess of companions is related to AGN activity or to some concurrent factor. When the proper adjustments are made a weak excess of about two in faint companions is found⁸⁴. In recent work⁸⁵ the environments of Seyferts and non-Seyfert Markarians were compared using galaxy counts from the Palomar Sky Survey. The number of companions found was essentially the same. Thus the excess observed by Dahari seems to be a reflection of membership in the Markarian list rather than AGN activity. In fact, Seyfert nuclei seem to be under-represented in the sample of strongly interacting (deformed) galaxies^{86,87}, and recent analysis using colours determined by the Infrared Astronomical Satellite (IRAS) confirms that close companions lead more frequently to a starburst galaxy than to an active galactic nucleus⁸⁸.

Because galaxies possess internal degrees of freedom, their collisions often are inelastic and can result in capture, leading

to mergers, which will be highly dissipative, if gas is present^{61,89,90}. The fate of a satellite on a decaying orbit and the effect it produces by imposing a prolonged torque is not to be confused with the outcome of a brief encounter. Some indication that Seyfert activity may be enhanced in very close, presumably bound, pairs of galaxies comes from the Keel *et al.*⁹¹ sample of 25 such galaxies. His complete sample of interacting galaxies shows a marginal 2σ effect. Given that the perturber itself is dense enough to avoid being dispersed, the amplitude of perturbation imposed on the stellar disk will steadily grow. A merger with a companion initially on a prograde orbit will excite the $m = 2$ mode in the disk, and this mode in turn will dominate the angular momentum transfer in the gas. In the case of a retrograde merger the initial symmetry of the galaxy would also be strongly affected, but we expect that chaotic orbits would become dominant. Of course, gas cannot be kept on intersecting orbits and will lose its angular momentum in cloud–cloud collisions or in interaction with the collective (stellar) gravitational field. The new equilibrium can be reached only once the satellite is disrupted or has sunk to the dynamical centre of the system.

The gas distribution before an encounter phase or at the onset of a global instability in the disk may be an important factor in the enhancement of circumnuclear activity. Estimates of the effect of inward propagating cloud–cloud collisions⁹² and density-wave focusing⁹³, as well as numerical simulations^{69,70,90} have used late-type galaxies, which have a centrally peaked distribution of molecular and atomic gas. Seyfert nuclei are, however, predominantly associated with early-type spirals. The latter frequently exhibit a central depression in distributions of atomic and/or molecular hydrogen more prominent in high-luminosity large-bulge systems^{94,95}. Could the action of a large-scale shock associated with the bar contribute to the formation of these 'holes' in the gas distribution, for instance, by inflow or conversion of atomic to molecular hydrogen (as occurs in spiral arms)?

The association of bars and Seyferts has been known for some time^{96,97}, and there is growing evidence that a large fraction of SBGs host bars^{98,99}. The detection of a bar is not trivial: modern optical techniques (including CCD imaging, which normally yield the most detailed morphological information), may fail to reveal even a strong bar¹⁰⁰. Because of the old stellar population and/or dust obscuration, near-infrared imaging of a bar seems to be crucial for its detection (see, for example, ref. 101 for NGC1566 and ref. 102 for NGC1068). This may explain why the rings, which are intrinsically related to bars and consist of a chain of H II regions and young stars, seem to be more frequently observed than bars themselves^{2,97}.

Nuclear activity in elliptical galaxies increases markedly with bolometric luminosity¹⁰³ and with a diminishing degree of rotational support in the stellar component^{104,105}. Evidence has also been accumulating that signs of interaction for elliptical increase with their radio luminosity^{106,107}. When traced to high redshifts, $z > 1$, host galaxies of powerful radio sources become unusually blue. Their 'multimodal' structure was initially claimed to be the result of ongoing mergers, but alternative models are probably more viable.

Dynamically, giant elliptical galaxies are similar to bars, but differ in degree of triaxiality and slow rotation. The kinematics of the gas also differ from that of disk galaxies. When found in the circumnuclear regions of normal and radio-loud elliptical galaxies, the gas often exhibits rapid rotation^{105,108} and distinct velocity reversals¹⁰⁹. The latter are indicative of radial flow and are also observed in barred spiral galaxies. The rotation axes of the gas and stars differ, but whether this is a reflection of the external origin of the gas or the result of underlying triaxial potential is as yet unclear. The first direct evidence for infall in these objects has been obtained recently from H I absorption¹¹⁰, but the source of the gas remains unknown. In galaxies with cooling flows, this infall may come from the gas condensing

and joining the observed disk at smaller radii, although the presence of dust is not consistent with this explanation¹¹¹.

Direct observations of QSO host galaxies are very limited and searches for possible environmental triggers of nuclear activity are hampered by the large dynamical range required. Nevertheless, several optical and infrared studies of low-redshift QSOs have produced claims that many of them reside in groups or small clusters of galaxies and are strongly interacting and possibly merging^{112–114}. QSO galaxies seem to be systematically more luminous than normal galaxies and Seyferts. Morphological peculiarities, seen in one-third of them, seem to be independent of the QSO luminosity¹¹⁵. Some indication that host galaxies experience bursts of star formation comes from long-slit spectroscopy¹¹⁶, and large amounts of molecular gas have been detected¹¹⁷. Although it is premature to analyse the relative importance of different triggers of QSO activity, there is no need, on the basis of existing data, to assume qualitatively different mechanisms for fuelling compared to these already discussed.

It is, at present, unclear to what extent AGN and SBG phenomena overlap and whether they are causally related. At a low level of activity they anticorrelate⁷⁸. At a high level, the far-infrared spectra of Seyferts show thermal emission from a starburst in addition to a non-thermal nuclear component^{118,119}. This is especially pronounced in Seyfert 1 galaxies^{120,121}. Together with the measured excess of CO emission (by a factor of about two), this may be evidence for the evolutionary sequence SBG → Seyfert 2 → Seyfert 1, providing a missing link between SBG and AGN¹²¹. A similar trend, SBG → QSO, has been suggested for the ultraluminous IRAS galaxies¹²².

Bridging the gap

A model in which large-scale non-axisymmetric perturbations cause the radial inflow that fuels the SBH does not address the whole complexity of the situation. First, about half of the spirals are barred or show oval distortions but only a few per cent of them host luminous AGN. Second, the inflow generated by a rotating bar potential does not extend all the way in to scales where turbulent viscosity or cloud–cloud collisions could take over. To overcome these difficulties it was suggested²⁷ that the gas accumulated in the central few hundred parsecs in the form of a rapidly rotating disk becomes gravitationally unstable again and forms a *gaseous* bar, which causes further inflow. This second stage may be common to both elliptical and spiral galaxies. The necessary conditions for the development of this instability is that the gas must represent a substantial fraction, $\geq 20\%$, of the dynamical mass and must be cool enough. Such concentrations of molecular hydrogen have been observed in SBG and Seyferts^{123–125}. The circumnuclear regions of Sérsic and Pastoriza galaxies (all barred at some level) were recognized as unusually bright and disturbed more than twenty years ago. Recent data indicate that, at least in Seyfert galaxies, molecular hydrogen is arranged in rotating disks coplanar with H I/stellar disks¹²¹. When resolved, they show strongly non-axisymmetric features interpreted as molecular bars^{125–128}.

According to the above ‘bars within bars’ model, the global gravitational disturbance—a dynamically unstable sequence of nested gaseous bars—drives inflow over a period of a few dynamical timescales. At each radius, the mass of the gas is some significant fraction of the total mass inside that radius. It was argued¹²⁹ that further inflow will create a totally self-gravitating gas cloud, leading to the formation of a dense stellar cluster. In our view, bar-driven inflow is more likely to slow down when the flow reaches radii where the mass inside r ceases to vary strongly with radius. In a nucleus with a SBH at the centre, this is probably the radius at which the SBH begins to dominate the gravitational potential, $\sim 40(M_{\text{SBH}}/10^8 M_{\odot})v_{\text{c},100}^2$ pc. At this point the flow is supported vertically (heated up) by the local gravitational instability, whereas the hose-pipe instability (or cloud–cloud collisions) limits the anisotropy in Δv

between the vertical and radial directions. When Δv is an appreciable fraction of the local keplerian velocity, $\geq 0.3v_{\text{c}}$, the inflow rate due to the cloud–cloud collisions can match the rate of inflow dictated by instabilities on larger scales. We speculate that this transition in the behaviour of the potential also causes a transition from bar-driven inflow to inflow driven by cloud–cloud collisions, described above. The process of gaseous inflow is probably accompanied by some star formation. In some cases circumnuclear star formation may even suppress the second stage of instability (the formation of a gaseous bar) by consuming the available gas. The kinematics of this ‘residual’ stellar population can bear the signature of its origin.

Unified model for fuelling AGN

Our analysis leads us to propose a specific description of the accretion flows that fuel luminous AGN in both spiral and elliptical galaxies. The main components are bar-driven inflow at large r , triggered by a number of factors both internal and external, and a marginally self-gravitating disk of clouds at smaller r (Fig. 2). Much work needs to be done on the cloud physics and on the transition from bar-driven to cloud-collision-driven (or magnetically driven) inflow. A dense star cluster cannot be ruled out theoretically, but it is not necessary and may be bypassed by evolution.

The ‘disk of clouds’ model has several features that may produce observable characteristics of AGN. If the clouds are optically thick and have a small covering factor, they may be resistant to the rapid X-ray ablation^{53,59,130}. The ability of inflowing gas to avoid dispersal by X-ray heating is necessary for the survival of the accretion flow, because the ablation rate cannot exceed the accretion rate. The thick part of the inflow could produce opaque molecular tori inferred to exist in Seyferts^{2,59,130,131}, Seyferts 1 and QSOs¹¹⁷ and may contribute to a thermal infrared spectral component in Seyferts 1 and QSOs^{36,37}. The clouds may also contribute to the production of the observed emission lines.

Observational and theoretical prospects

The details of the flows that fuel AGN can best be elucidated by observing their morphologies and kinematics. Multifrequency studies are needed to highlight different aspects of these flows and their triggers. Ground-based surveys for faint companions of galaxies (dwarf ellipticals and Magellanic irregulars) may help to resolve the controversy over the role of encounters in triggering activity. Surveys of faint companions of luminous active galaxies at high redshifts could show whether the evolution of nuclear activity with redshift is directly related to the frequency of encounters, or has some other, presumably intrinsic, cause. High-resolution morphological and kinematic studies of H I and CO emission (radio) and of H II regions (optical and ultraviolet) may determine whether nonaxisymmetric flows in a cold gaseous disk—gaseous bars—are responsible for inflow on scales between ≤ 1 kpc and a few tens of pc. Because they involve the pre-existing stellar population, stellar bars are most readily detectable in the near-infrared; improved array detectors on ground-based and airborne telescopes may help determine the incidence of stellar bars in active galaxies, and their morphological relationship (such as position angle) relative to the gaseous structures and star formation regions. There is also a need for a new study that uses our improved knowledge of how to recognize oval disks in order to look at the relative incidence of bars, ovals and companions in well-defined samples of active galaxies.

Infrared imaging and spectroscopy in the far-infrared, necessary to study ‘buried’ star formation regions and the reprocessed radiation from the active nucleus, will have to await the next generation of space-based observatories, although important submillimetre observations can be done from the ground. Far-infrared and submillimetre imaging is particularly important for determining whether the emission in these bands, often a

significant fraction of the total output of an AGN, arises directly from non-thermal emission in the nucleus, or because of reprocessing of nuclear emission in the dusty ISM^{36,60}.

Ultraviolet and optical imaging observes those regions of gas that are directly illuminated by the hard nuclear radiation. Studies^{113,132,133} show that the irradiated regions are patchy, or confined to a restricted solid angle as viewed from the nucleus. This type of observation gives clues about the covering factor and size of opaque clouds in the ISM, and to the intrinsic collimation of the central source. Spectroscopic observations tell us whether the irradiated gas is participating in the general rotation of the galaxy, or is disturbed by either the central engine or by gravitational torques. The Space Telescope will allow these observations on scales as small as a few tens of parsecs in nearby Seyfert galaxies.

X-ray observations will be necessary to determine the role of 'hot' gas in fuelling AGN. ROSAT (Röntgensatellit) will add to our catalogue of 'cooling flows', and may also provide the first rudimentary maps of X-rays scattered by circumnuclear hot gas (such as that in an X-ray-heated wind). The vastly improved imaging and spectroscopic capabilities of AXAF (Advanced X-ray Astronomy Facility) and XMM (X-ray Multi-mirror Mission), respectively, will enable detailed maps of cooling flows to be made for the first time. We should be able to determine whether some spiral galaxies, as well as many ellipticals, possess cooling flows. This information is important for our understanding of fuelling because Seyfert galaxies are generally identified as spirals. Spectroscopy of iron K α is an important diagnostic of X-ray-irradiated gas obscured by dust in the central region of an active galaxy¹³⁴. X-ray polarimetry will provide another probe of the scattering of X-rays by hot gas in the 'intermediate' zone of AGN^{130,135}.

Indirect means will have to be used to determine the structure of inflow on scales smaller than a few tens of parsecs. Such techniques as speckle interferometry, very-long-baseline interferometry and medium-baseline radio interferometry, may provide some useful morphological information on scales down to a few parsecs or less. Other techniques which may yield clues to the geometry of the inner flow include spectropolarimetry and spectroscopy of X-ray fluorescence lines and high- J (rotational quantum number) molecular lines¹³⁶, expected from the irradiated dense gas in these regions.

Further theoretical advances require increasingly sophisticated numerical simulations coupling stellar and gas dynamics, which might help us to answer such questions as: how long-lived is the observable response of a galaxy to a tidal encounter? How difficult is it to trigger a runaway bar instability in the gas? How does the initial distribution of stars and gas affect the fuelling process? The greatest challenges are the inclusion of adequate dynamic ranges in both space and time. Tree codes (hierarchical N -body codes) seem well suited to this purpose, and adaptive mesh techniques may also prove important. In addition to refining the simulation of 'global' processes, more realistic models are required for such 'microscopic' processes as cloud formation and cloud-cloud collisions. These processes may best be investigated using high-resolution gas-dynamics codes that include radiative losses and transport properties. The possible dynamical importance of magnetic stresses requires the use of codes incorporating full magnetohydrodynamics, which are becoming available. Unfortunately, realistic simulations of the occurrence and effects of star formation will probably not be available for some time. □

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ARTICLES

A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo

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The murine *oct-3* gene encodes a transcription factor containing a POU-specific domain and a homeodomain. In marked contrast to other homeodomain-encoding genes, *oct-3* is expressed in the totipotent and pluripotent stem cells of the pre-gastrulation embryo and is down-regulated during differentiation to endoderm and mesoderm, suggesting that it has a role in early development. The *oct-3* gene is also expressed in primordial germ cells and in the female germ line.

THE vertebrate homeobox genes studied so far are expressed after the primitive streak stage of development in temporally and spatially restricted patterns that suggest roles in segmentation, anterior-posterior axis determination and cell type

specification¹. As an approach to studying transcription factors active in earlier stages of mammalian development, before cell type determination, we have cloned the structural gene encoding NF-A3, a nuclear factor specific for the regulatory octamer DNA motif, which is selectively expressed in undifferentiated embryonal carcinoma (EC) cells (refs 2, 3; NF-A3 is the factor termed Oct-4 in ref. 3). EC cells, which are derived from the stem cells of teratocarcinomas⁴⁻⁷, can be induced to differentiate *in vitro*, and during this process NF-A3 expression decreases^{2,3}. Furthermore, the octamer DNA motif to which NF-A3 binds is functional in undifferentiated EC cells and in the blastocyst^{2,8}.

Cloning of *oct-3*

NF-A3 has DNA-binding characteristics indistinguishable from those of two other octamer-binding proteins, Oct-1⁹⁻¹⁴ and Oct-2¹⁵⁻²⁴, both of which use a homeodomain to bind DNA². To clone NF-A3, we screened a complementary DNA library prepared from undifferentiated F9 EC cell⁵ messenger RNA with a probe from the homeobox region of *oct-2* at lowered stringency. All cDNAs isolated were from the same murine gene, which we term *oct-3*. The sequence had a long open reading frame encoding 352 amino acids (Fig. 1a, b).

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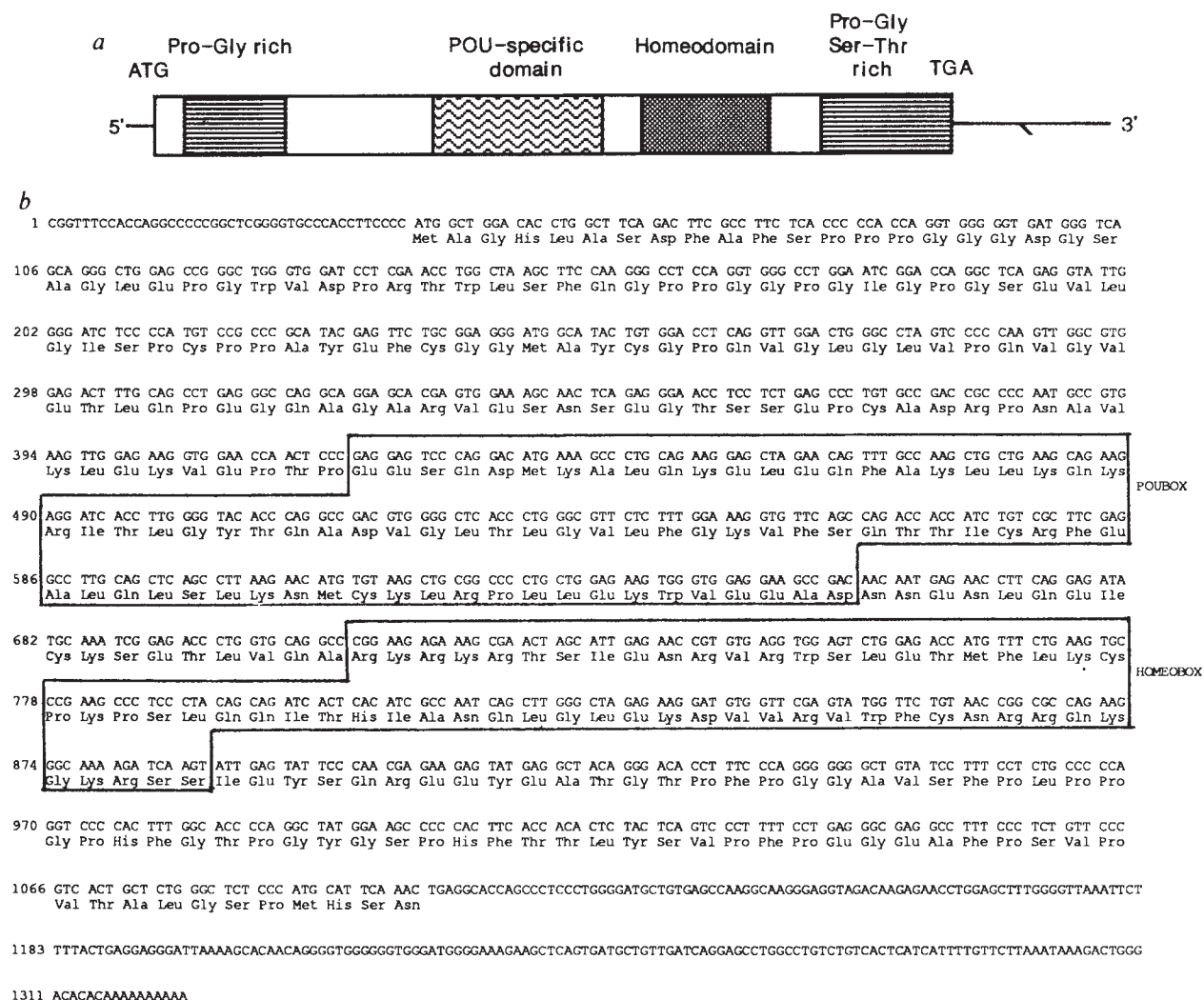


FIG. 1 Structure of the Oct-3 protein. *a*, Schematic of *oct-3* cDNA. *b*, DNA and corresponding amino-acid sequence of *oct-3*.

METHODS. Poly(A)-containing mRNA from undifferentiated F9 EC cells was used with oligo(dT) primers to prepare cDNA³⁷ which was ligated into λ ZapII phage arms (Stratagene). Replica filters were screened with a ³²P-labelled 200-base-pair (bp) *Hae*III fragment of the *oct-2* cDNA encompassing the *oct-2* homeobox²⁰. The filters were hybridized using standard conditions³⁸

and washed in 2 $\times$ SSC, 0.1% SDS at 60 °C. Positive phage (1/1,000 plaque-forming units (PFU)) were plaque-purified and converted to Bluescript plasmids containing the cDNA insert according to the manufacturer's protocol (Stratagene). The *oct-3* inserts from 32 plasmid DNAs were sequenced on both strands by the dideoxynucleotide method, using Sequenase polymerase (USB)³⁹.

The predicted Oct-3 protein is a new member of the subfamily of homeodomain proteins that also contain a POU-specific domain (the so-called POU-domain proteins)²⁵. Oct-3 differs from all previously cloned POU-domain proteins at several conserved amino-acid positions in the POU-specific domain, in the spacer region between the POU-specific domain and the homeodomain, and in the remainder of the protein. The N terminus of Oct-3 contains a region rich in proline and glycine residues, and the C terminus contains a region rich in proline, glycine and serine/threonine residues. Proline-rich regions can function as transcriptional activator domains²⁶.

Oct-3 is a transcription factor

To test whether *oct-3* encodes an octamer-binding protein, the *oct-3* cDNA was transcribed and translated *in vitro* and tested for its ability to bind to octamer-containing DNA probes in a mobility-shift DNA-binding assay (Fig. 2). The *oct-3* translation products gave one major (Oct-3A) and one minor (Oct-3B) shifted band using a wild-type octamer probe. Both *oct-3* translation products were specific for the octamer motif as they were not seen using a mutant octamer probe and their binding could be competitively inhibited by unlabelled wild-type, but not

mutant, octamer-containing oligonucleotides. It is of interest that Oct-3A co-migrated with the NF-A3 band in extracts from EC cells. The relative molecular mass of NF-A3 was determined to be 42,000 (42K) by recovery of NF-A3 from an SDS polyacrylamide gel (ref. 27; data not shown). SDS-PAGE of internally labelled *in vitro* translation products of *oct-3* showed a major 42K species and a minor 35K species (data not shown). It thus seems likely that the *oct-3* gene encodes NF-A3.

To test whether the Oct-3 DNA-binding protein functioned as a transcription factor, *oct-3* cDNA was cloned into a mammalian expression vector and co-transfected with an octamer-dependent reporter plasmid into HeLa cells (Fig. 3). An RNase protection assay of RNA from the transfected cells showed that the *oct-3* expression vector stimulated correctly initiated mRNA synthesis from the reporter plasmid containing a wild-type octamer DNA motif (Fig. 3, lane 3). Mutation of the octamer motif substantially reduced the transactivation (Fig. 3, lane 4).

Expression in the early embryo

The *oct-3* mRNA was detected on northern blots of undifferentiated EC (Fig. 4a, lane 1) and embryonic stem (ES) cell RNA (Fig. 4b) as a 1.55-kilobase (kb) species. No *oct-3* expression

FIG. 2 The *oct-3* cDNA encodes an octamer-binding protein. Protein synthesized *in vitro* was analysed in a mobility shift DNA-binding assay using either wild-type (wt) or mutant (mut) kappa promoter (kprom) probes¹⁵. Lanes 1–6, protein synthesized from *oct-3* sense mRNA; lane 7, protein synthesized from *oct-3* antisense mRNA; lanes 8–13, F9 whole cell extracts; lanes 13–15, whole-cell extracts from Ntera2/D1 cells. Unlabelled competitor DNAs were wild-type and mutant kprom, and wild-type and mutant IgH gene enhancer (enh) oligonucleotides (see Methods). The faint band migrating with Oct-1 that was seen using translation products derived from both sense and antisense *oct-3* mRNA was derived from the reticulocyte extract.

METHODS. mRNA synthesized *in vitro* was made from the *oct-3* cDNA in the Bluescript SK-plasmid (Stratagene). The plasmid was linearized and sense mRNA made using T7 polymerase. Antisense mRNA was made using T3 polymerase (Stratagene). The mRNA (1 µg) was used for *in vitro* translation reactions in rabbit reticulocyte lysates (Promega). Of a 50 µl total reaction, 4 µl was used for analysis in mobility-shift DNA-binding assays. Whole-cell extracts from Ntera2/D1⁷ and F9^{4,5} cells were made according to the procedure of Zimarino *et al.*⁴⁰. Mobility-shift DNA-binding assays for octamer-binding proteins were performed as previously described¹⁵. Oligonucleotides used as probes and competitive inhibitors were the following:

```
wt kprom    GATCTAACTGCTTCTTAATTGTCATACCCCTCACTGCATCG
             ATTGACGAAGAATTAAACGTATGGAGTGACGTAGCCTAG
mut kprom   GATCTAACTGCTTCTTACAAGTGTGACCCCTCACTGCATCG
             ATTGACGAAGAATGTTCACACTGGGAGTGACGTAGCCTAG
wt IgH enh  CGAGCCCGGGTAATTGCAATTTCTACTAG
             TOGAGCTCGGGCCCCATTAACGTAAAGATGATCAGCT
mut IgH enh  CGAGCCCGGGTATGAGTGTGTTCTACTAG
             TOGAGCTCGGGCCCCATACTCACACAAGATGATCAGCT
```

Probes were labelled using the Klenow fragment of DNA polymerase I (New England Biolabs) with all four ³²P-labelled nucleotides (Amersham)³⁸. Competition experiments included 9 ng unlabelled oligonucleotides in the binding reactions.

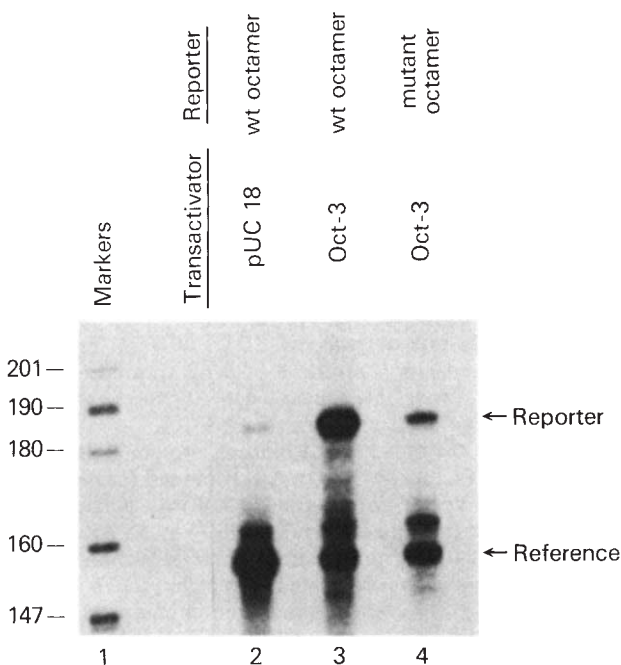
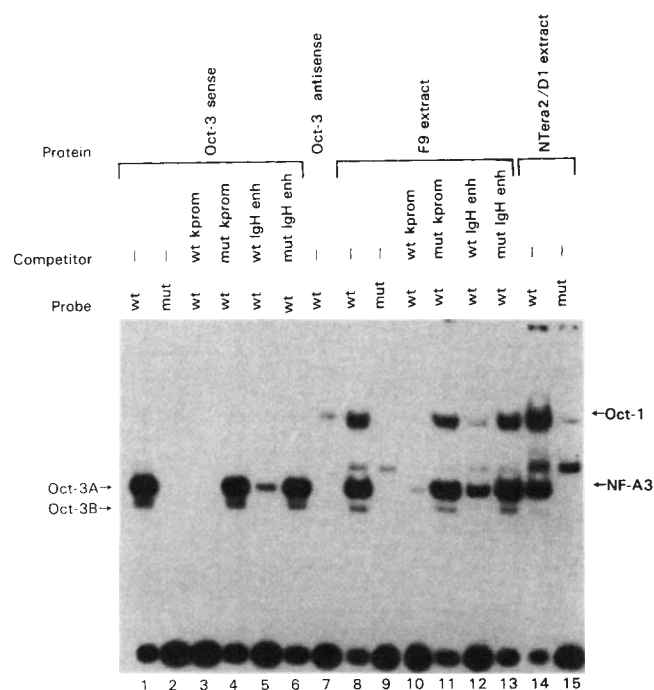


FIG. 3 Oct-3 transactivation of an octamer-dependent promoter. HeLa cells were co-transfected with three types of plasmids: (1) a transactivator plasmid expressing *oct-3* (lanes 3, 4) or control pUC18 plasmid (lane 2); (2) a reporter plasmid containing either a wild-type octamer motif (lanes 2, 3) or a mutated octamer motif (lane 4) cloned upstream of the TATA box of the rabbit β -globin gene; (3) a reference plasmid to monitor transfection efficiency (lanes 2–4). Cytoplasmic mRNA from the transfected cells was analysed using an RNase protection assay. The protected bands corresponding to the mRNAs derived from the reporter plasmid and the reference plasmid are indicated. The residual transactivation seen with the mutated octamer reporter construct was also observed with an *oct-2* expression vector (data not shown) and may be due to weak binding of the octamer-binding proteins to the mutated motif. Lane 1 contains DNA size markers of the number of base pairs indicated.

METHODS. The transactivator plasmid was constructed by cloning the *NcoI*–*BclI* fragment of the *oct-3* cDNA into a mammalian expression vector derived from pSTC-X556⁴¹, in which the cDNA is expressed from the cytomegalovirus enhancer–promoter and herpes simplex virus thymidine kinase gene leader. The reporter plasmids (ref. 23; Fig. 6b, plasmids 4 and 5) and reference plasmids have been described²³. HeLa cells were transfected using the calcium phosphate precipitation method using 3 µg transactivator or control plasmid, 15 µg reporter plasmid and 2 µg reference plasmid as described²³. Cytoplasmic mRNA was analysed by RNase protection²³.

was seen in EC cells differentiated *in vitro* using retinoic acid (Fig. 4a, lane 2). As ES cells are totipotent stem cells derived from the inner cell mass (ICM) of murine blastocysts²⁸⁻³¹, we next studied the expression of *oct-3* in the developing embryo by *in situ* hybridization. The mRNA was detectable in oocytes (Fig. 5a, b); a similar level was found in fertilized ova at 0.5 days post coitum (d.p.c.) (data not shown). The mRNA was again detected in the 2.5 d.p.c. morula (Fig. 5c, d), and in the blastocyst at 3.5 d.p.c.; *oct-3* mRNA was detected in both the trophectoderm and, at distinctly higher levels, in the ICM cells (Fig. 5e, f). The increase in *oct-3* mRNA expression between 2.5 and 3.5 d.p.c. suggests that the zygotic *oct-3* gene is activated before 3.5 d.p.c. In hatched blastocysts, at 4.5 d.p.c., the mRNA was no longer detectable in trophectoderm cells but was strongly expressed in the epiblast and present at an intermediate level in the primitive endoderm (Fig. 5g, h). After implantation, in the 5.5 d.p.c. egg cylinder, *oct-3* mRNA was present only in the primitive ectoderm, there being no detectable expression in the extra-embryonic cells derived from the trophectoderm (Fig. 5i).

At 7 d.p.c., the mesoderm of the primitive streak begins to differentiate from the primitive ectoderm. This differentiation is accompanied by a down-regulation of *oct-3* expression as a lower signal was detected over the primitive ectoderm-derived mesoderm cells (Fig. 5j, k, l, m). The ectoderm cells at this time, and at 8.5 d.p.c. (Fig. 5n, o), expressed *oct-3*. The differentiated descendants of the primitive endoderm, the visceral endoderm, showed no *oct-3* expression. After this stage in development, we observed no expression in somatic cells (data not shown), a conclusion supported by the results of the RNase protection analysis in Fig. 6b (lane 7), which shows that no *oct-3* mRNA could be detected in the whole embryo at 14 d.p.c.

Expression of *oct-3* was, however, observed in primordial germ cells in the mid-gestation embryo. At 10.5 d.p.c., cells expressing *oct-3* were identified as migrating primordial germ cells by virtue of their alkaline phosphatase staining (Fig. 5q, r). At 11.5 d.p.c., the primordial germ cells in the genital ridges clearly express *oct-3* (Fig. 5s, t).

Expression in maturing oocytes

In the adult, *oct-3* mRNA expression was detected in both ovary and testis (Fig. 6a) but not in brain, heart, liver, spleen, skeletal muscle, lung, kidney or pancreas (data not shown). An RNase

protection assay of *oct-3* mRNA expression revealed two protected species using ovary mRNA (Fig. 6b, lane 4). One was of 442 base pairs, the same size as the fully protected species seen using F9 EC cell mRNA (Fig. 6b, lanes 1, 2), and the other was of 380 base pairs. The 442-bp species protected by *oct-3* was also detected using adult testis mRNA (data not shown).

Having seen, by *in situ* hybridization, *oct-3* mRNA expression in oocytes, we investigated *oct-3* expression during oocyte maturation. Messenger RNA prepared from 200 oocytes in different stages of maturation³² was hybridized on a northern blot with the *oct-3* probe. The *oct-3* mRNA was detectable in maturing and ovulated oocytes but not in resting oocytes (Fig. 6c).

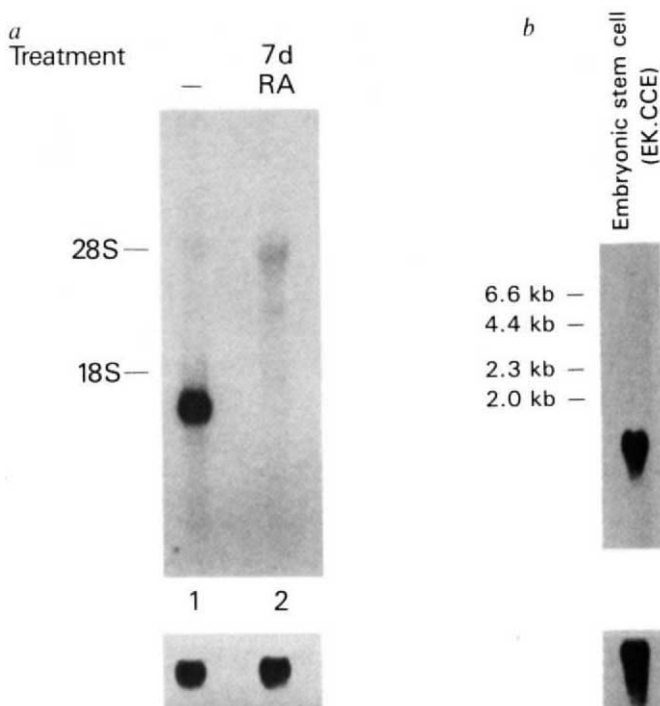
Discussion

The *oct-3* gene encodes a transcription factor, containing a POU-specific domain and a homeodomain, that is expressed in undifferentiated pluripotent cells of the early embryo and in germ cells. Like Oct-1⁹⁻¹⁴ and Oct-2¹⁵⁻²⁴, Oct-3 positively regulates transcription by way of the octamer DNA motif. In ES cells and in the ICM of the blastocyst, the octamer motif functions as a strong positive regulatory motif⁸. In undifferentiated EC cells, the octamer motif functions both as a positive and a negative transcriptional regulatory motif, depending on the DNA context in which it is tested^{2,8}. Our results show that Oct-3 could account for the positive transcriptional activity of the octamer motif in EC and ES cells and in the ICM of the blastocyst. It is unclear whether Oct-3 or another factor in EC cells is responsible for the negative transcriptional activity of the octamer motif in these cells.

The feature common to all of the cells expressing *oct-3* in the early embryo is that they retain the capacity for differentiation along multiple lineages. Blastocyst chimaera experiments have demonstrated the totipotent nature of the ICM cells³³. The trophectoderm differentiates into a variety of extra-embryonic cell types, including the fetal contribution to the placenta. The primitive ectoderm of post-implantation embryos gives rise to all of the somatic and germ-line cells of the developing fetus. By contrast, previously cloned homeobox genes are expressed later in post-gastrulation embryos in cells that have begun to differentiate into more committed cell types¹. Thus, the function of Oct-3 in mammalian development must be distinct from that

FIG. 4 Expression of *oct-3* mRNA. a, Northern blot analysis of *oct-3* in Ntera2/D1 cells untreated (lane 1) and treated with 10^{-5} M retinoic acid (RA) for 7 days (7d) (lane 2). b, Northern blot analysis of *oct-3* expression in ES cells. All blots were rehybridized with human α -actin to control for levels of RNA, as shown in the lower panel⁴².

METHODS. Northern blots were performed as described³⁸. Samples (15 μ g) of total cellular RNA were assayed from each cell line. ³²P-labelled DNA from the 3' end of *oct-3* (an *A*haII-*E*coRI, 442-bp fragment) was used as probe. Blots were hybridized using standard conditions³⁸ and washed at 60 °C in $2 \times$ SSC, 0.1% SDS. Autoradiography was for 24 h at -70 °C with an intensifying screen.



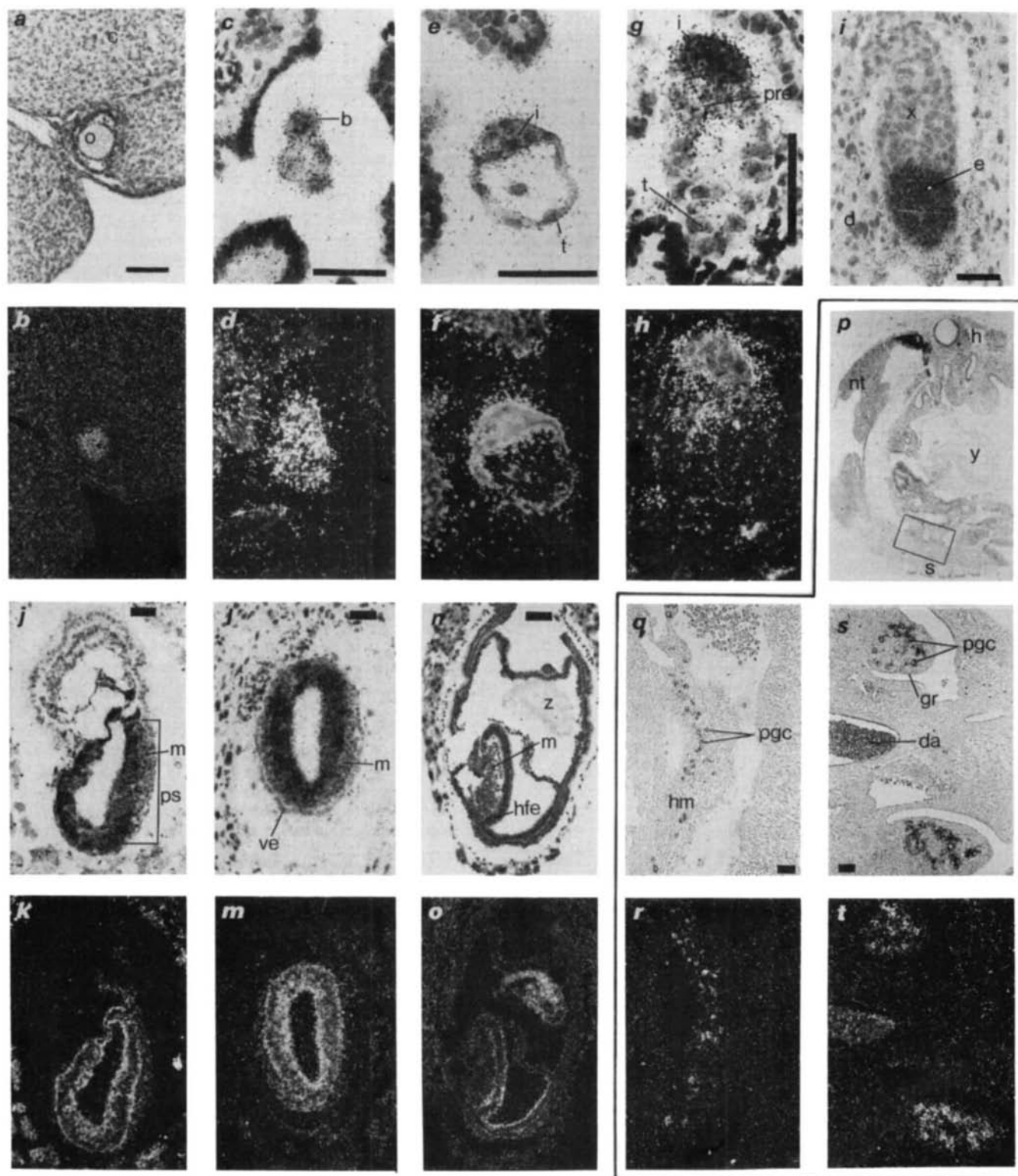


FIG. 5 *In situ* hybridization of ^{35}S -labelled antisense RNA probe for *oct-3* to sections of mouse embryos. *b, d, f, h, k, m, o* and *r*, Darkfield photographs of the sections shown under brightfield illumination in *a, c, e, g, i, l, n* and *p*, respectively. *a, b*, Oocyte within ovary; *c, d*, 2.5 d.p.c. (morula stage) embryo within oviduct; *e, f*, 3.5 d.p.c. blastocyst within oviduct; *g, h*, 4.5 d.p.c. hatched blastocyst within oviduct; *i*, 5.5 d.p.c. egg cylinder stage embryo within its deciduum; *j, k*, longitudinal, and *l, m*, transverse, sections of 7.5 d.p.c. primitive streak stage embryo; *n, o*, 8.5 d.p.c. embryo. In this section, chosen because it shows the head-folds clearly, there is nonspecific probe absorption to an area of acellular material labelled *z*. The cells of the allantois (not shown) are equivalent to other mesoderm in signal intensity. *p*, 10.5 d.p.c. embryo hybridized to the *oct-3* probe; *r*, higher magnification of box in *p*; *q*, near-

adjacent section to that shown in *p* and *r*, stained for alkaline phosphatase; *s*, 11.5 d.p.c. embryo stained for alkaline phosphatase; *t*, adjacent section to *s*, hybridized to the *oct-3* probe. The signal over the dorsal aorta results from nonspecific probe absorption. Labels are: *b*, blastomere; *c*, corpus luteum; *d*, deciduum; *da*, dorsal aorta; *e*, embryonic ectoderm; *gr*, genital ridge; *h*, head; *hfe*, head-fold ectoderm; *hm*, hindgut mesentery; *i*, inner cell mass; *m*, mesoderm; *nt*, neural tube; *o*, oocyte; *pgc*, primordial germ cell; *ps*, primitive streak; *s*, somites; *t*, trophectoderm; *ve*, visceral endoderm; *x*, extraembryonic ectoderm; *y*, heart; *z*, acellular material within exocoelom. Bar, 50 μm .

METHODS. *In situ* hybridization was performed as described⁴³. The probe corresponded to nucleotides 865–1,325 of the *oct-3* cDNA (Fig. 1*a, b*). No

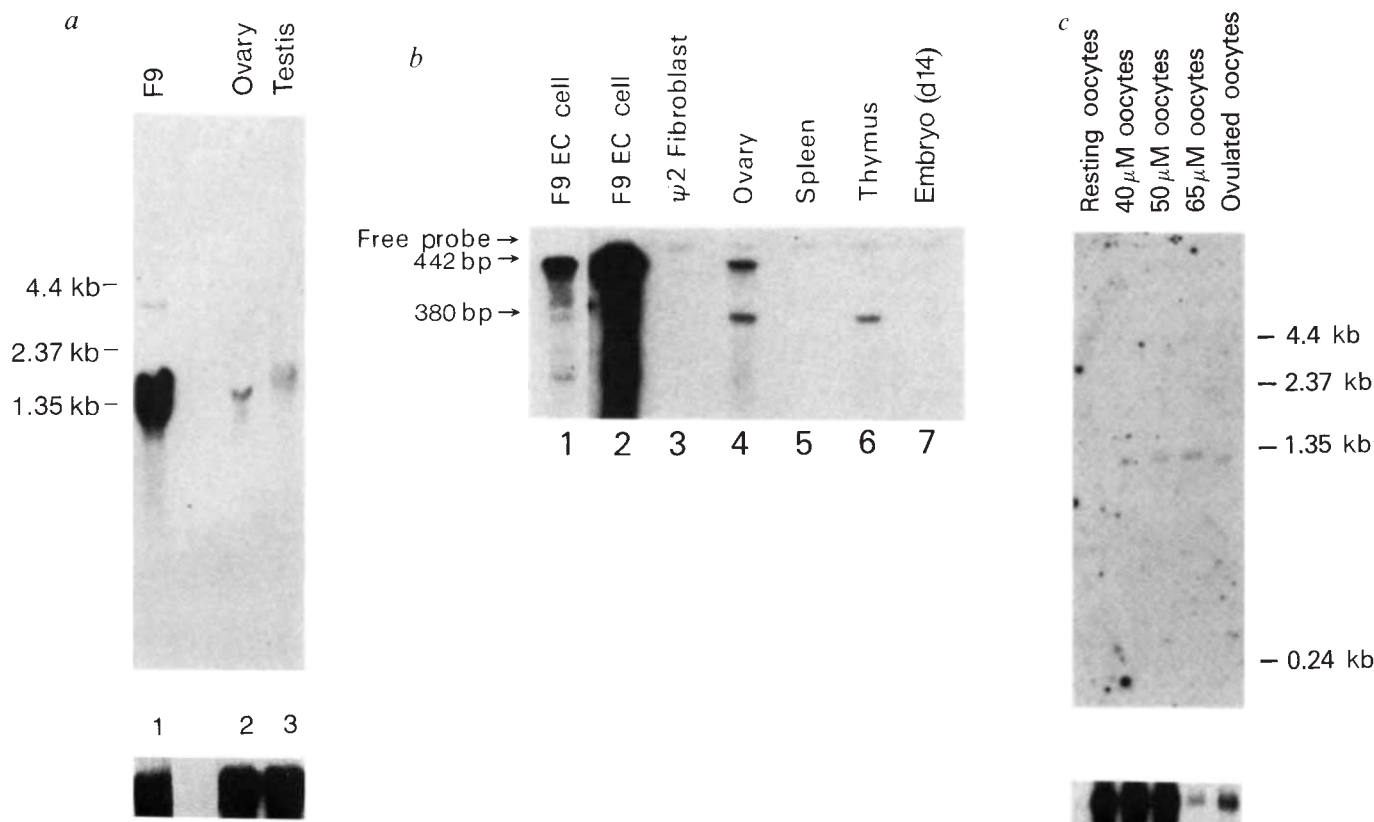


FIG. 6 *a*, Northern blot analysis of *oct-3* mRNA expression in murine adult tissues. Poly(A)⁺ mRNA (10 μg) was analysed from ovary (lane 2) and testis (lane 3). Lane 1 contains 15 μg total RNA from F9 EC cells. RNA size markers are indicated. *b*, RNase protection assay of *oct-3* mRNA expression. Lane 1 is from a 4-h autoradiograph and lanes 2–7 are from a 24-h autoradiograph of the RNase-protection gel. Lanes 1 and 2 show the analysis of 10 μg total F9 RNA. A sample (30 μg) of total cellular RNA was assayed from ψ2 (mouse fibroblast) (lane 3), ovary (lane 4), spleen (lane 5), thymus (lane 6) and total day-14 embryo (lane 7). Left, migration of the free (undigested) probe (503 bp), the fully protected probe (442 bp) and a smaller protected species of 380 bp. *c*, Northern blot analysis of *oct-3* expression in murine oocytes. RNA from resting oocytes (lane 1), growing oocytes (lanes 2–4) and ovulated oocytes (lane 5) was analysed. RNA size markers are on the right. Hybridization of the blots with human α -actin is shown in the lower panel.

METHODS. *a, c*, Northern blots were performed as described in Fig. 4. *c*, Oocytes in various stages of development were isolated according to the method of Huarte *et al.*³². Resting oocytes (~15 μM diameter) were collected from 3-day-old mice; 40 μM, 50 μM and 65 μM oocytes were collected from 7-, 14- and 21-day-old mice, respectively. Ovulated oocytes were from mice superovulated as described in ref. 32. Each lane contains RNA from 200 oocytes. The blot was autoradiographed at -70 °C with an intensifying screen for 5 days. *b*, RNase protection assay was performed as described⁴⁶. An *AhaII-EcoRI* (442 bp) fragment from the *oct-3* cDNA was cloned into plasmid pGEM-2 (Promega). The plasmid was linearized with *PvuII* and transcribed with SP6 polymerase in the presence of [³²P]CTP (Amersham). RNAs from tissues and cell lines were precipitated and hybridized for 18 h at 55 °C. Autoradiography was for 48 h at -70 °C with an intensifying screen. Abbreviation: d14, day 14.

of other homeodomain-containing proteins by virtue of its unique expression pattern in the early embryo.

As cells differentiate into more committed cell types, *oct-3* expression is consistently down-modulated. This phenomenon is observed during the *in vitro* differentiation of EC cells, during the formation of the trophectoderm and primitive endoderm of the late blastocyst and again as the mesoderm of the primitive streak arises from the primitive ectoderm. After the ninth day of gestation, *oct-3* expression is undetectable in somatic cells. These findings suggest that the down-modulation of *oct-3* is important for normal differentiation during embryogenesis.

signals above background were observed in sense-strand control hybridizations. Blastocysts (3.5 and 4.5 d.p.c.) were prepared by flushing from the mother's uterus and pipetting into the ampulla of another female who had mated 12 h previously. The oviduct containing blastocysts and 0.5 d.p.c. embryos was processed as a whole. For *q* and *s*, tissue preparation was according to an unpublished modification (A. McLaren, personal communication) of the procedure of Gabe⁴⁴. Alkaline phosphatase staining was according to Donovan *et al.*⁴⁵. All mice were CBA.

Germ cells, however, continue to express *oct-3*. Unlike previously studied homeobox genes, *oct-3* is selectively expressed in primordial germ cells and maturing oocytes¹. These data further suggest a tight connection between the expression of *oct-3* and a high degree of pluripotency as both primordial germ cells and oocytes³⁵ can, under certain experimental conditions, differentiate along several lineages. As *oct-3* is expressed in maturing, but not resting oocytes, it may transactivate genes important for oocyte maturation. In *Drosophila* embryogenesis, early zygotic gene expression is controlled, in part, by maternally derived transcription factors containing homeodomains³⁶. It will be particularly interesting to investigate whether maternally expressed Oct-3 positively autoregulates zygotic *oct-3* gene expression.

Note added in proof: Since the submission of this manuscript, two other groups have reported sequences that are likely to be derived from the same gene as *oct-3*^{47,48}. Our sequence agrees with that of Scholer *et al.*⁴⁸, except that their sequence seems to be 127 base pairs shorter at the 5' end, resulting in a protein that lacks 28 N-terminal amino acids. Our sequence differs from that of Okamoto *et al.*⁴⁷ in that it contains an additional G residue at position 924, resulting in a different reading frame at the C terminus. □

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LETTERS TO NATURE

On the close environment of BL Lacertae objects

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THE local environment of BL Lacertae objects, which resemble quasars but lack emission lines, is poorly understood. In the few cases where the surrounding nebulosity has been studied in detail, it is consistent with the presence of a giant elliptical galaxy¹, but the evidence that the BL Lac and the putative galaxy are physically associated rests solely on their positional coincidence. An alternative hypothesis, that BL Lacs are gravitationally lensed^{2,3} and that the surrounding emission is from the foreground lensing object, gains some support from a number of observations⁴⁻⁶ which reveal less than perfect alignment between BL Lacs and surrounding emission. We have begun a systematic programme of high-resolution imaging aimed at understanding in a general way the local environment of BL Lacs. Here we describe a first series of images, which show the presence of emission features around most of the BL Lacs observed. Typically, this emission is close (<5 arcsec) to the BL Lac, and faint ($m_R = 21$). We discuss the interpretation of these companions in terms of both interacting objects and gravitational lenses.

The observations were made with the 3.5-m New Technology Telescope⁷ of the European Southern Observatory (ESO) during its commissioning (see Table 1). This telescope has a thin primary mirror with actuators that can adjust the shape of the mirror to optimize imaging. Owing to an extremely well apodized point spread function (PSF), it can reveal weak features close to bright point-like sources, which is a key requirement for the imaging and spectroscopy study of the close environment of BL Lacs.

The second ESO Faint Object Spectrograph and Camera (EFOSC2), very similar to that installed on the observatory's 3.6-m telescope⁸, was used in image mode, with the R filter or white light.

During the first commissioning run the EFOSC2 was mounted without the adaptor/rotator so only very short exposures were possible for most positions in the sky and the active optics

system was disabled. The observations were made under good seeing conditions (≈ 1 arcsec). The absence of fine guiding resulted in a slightly distorted point spread function that varied from frame to frame. For frames in which sufficiently bright stars are present, we used a two-gaussian model to represent the core and wings of the point spread function in fitting the stellar images. In other frames, only the core component of the function was represented. The full width at half maximum (FWHM) of the core component in the x and y directions (in the frame of reference of the CCD detector) given in Table 1, allows a direct appreciation of the overall spatial resolution obtained in each frame. We obtained 12 frames for 8 BL Lacs. We are now obtaining images and spectroscopic data for a larger sample of BL Lacs. Details of the observations and results of the larger sample will be reported elsewhere.

The presence of close (<5 arcsec) companion objects is clearly detected in three sources: H0323+02, H1722+11 and PKS2155-30.

In H0323+02, a BL Lac with a remarkably large and rapid X-ray variability, a faint (apparent red magnitude, m_R , of 21.3) unresolved object, shows up clearly 2.3 arcsec southwest (position angle, $PA \approx 230^\circ$) of the centre (Fig. 1a). At the redshift attributed to the BL Lac ($z = 0.147$), the absolute magnitude would be $M_R \approx -18.6$ and the projected distance 8 kpc (using a Hubble constant H_0 of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and a cosmological constant q_0 of 0). Other features are also apparent: a complex

TABLE 1 Observations

Object	z	Date* (UT)	m_R	Filter	Exposure (s)	FWHM (arcsec)
						x, y
PKS0048-09	—	11.25	15.7	R	60	0.65, 0.62
		11.27		wl†	10	0.58, 0.67
PKS0118-27	0.56	12.24	15.9	R	120	0.74, 0.73
		12.25		R	30	0.87, 0.85
PKS0138-09	—	12.26	17.0	R	30	0.91, 0.88
H0323+02	0.147	11.35	16.4	R	120	0.89, 0.83
H0414+00	—	11.36	16.6	R	120	0.89, 0.92
H1722+11	—	11.04	15.3	R	120	0.92, 0.85
		11.06		R	30	0.95, 0.85
H2155-30	—	12.02	13.9	R	60	1.10, 0.92
		12.03		R	30	1.20, 1.30
H2351-31	0.17	11.37	17.1	R	120	0.68, 0.68

* In August 1989.

† wl, white light.

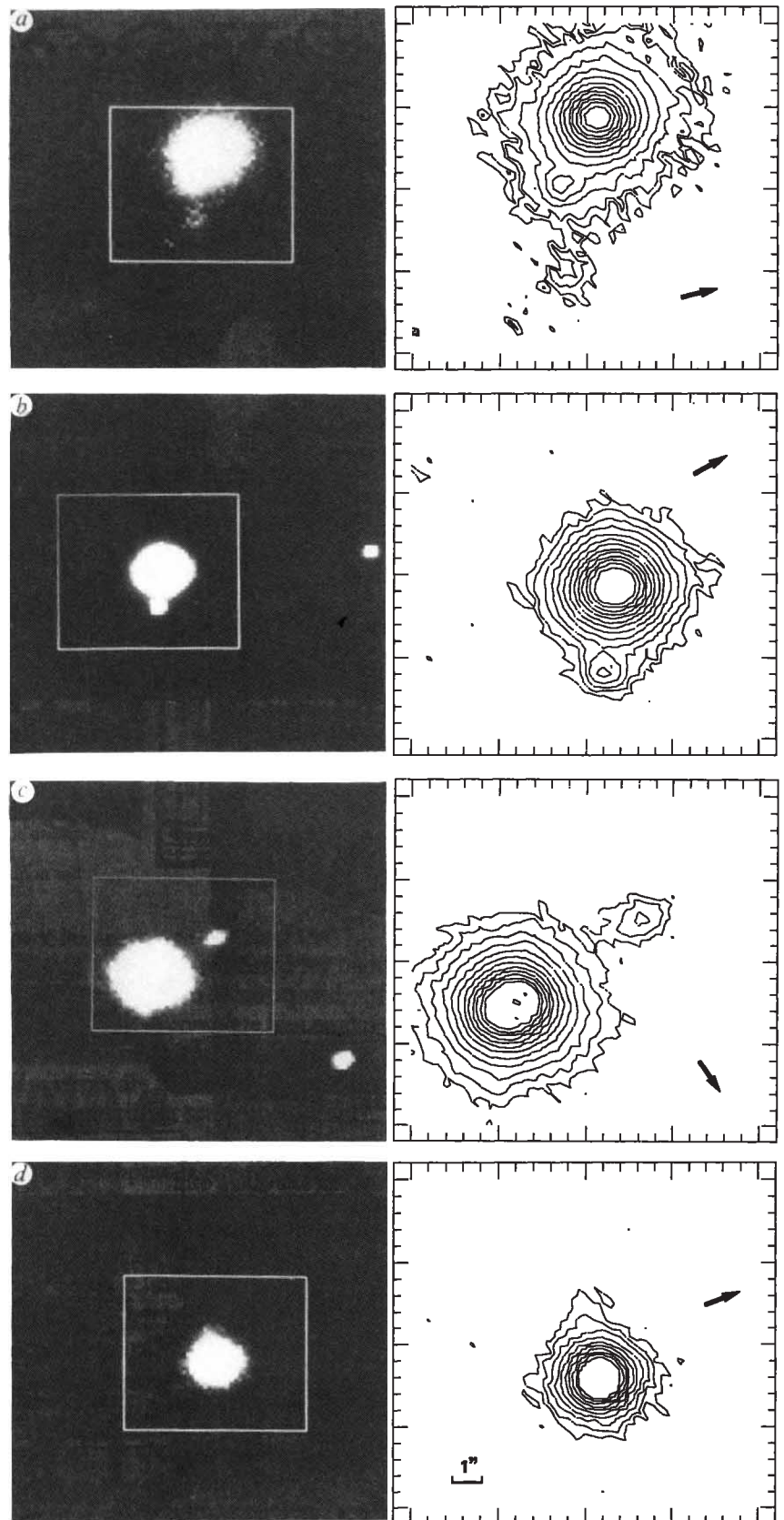


FIG. 1 On the left, central portions (22×22 arcsec) of CCD images of selected BL Lacs; the isophotal maps of the regions in white boxes are given on the right (spacing, 0.5 mag). Arrows point to the north; east is to the left of north. *a*, H0323 + 02 (lowest isophote: 22.9 mag arcsec $^{-2}$). *b*, H1722 + 11 (lowest isophote: 22.6 mag arcsec $^{-2}$). *c*, PKS2155 - 30 (lowest isophote: 22.5 mag arcsec $^{-2}$). *d*, PKS0048 - 09 (lowest isophote: 22.2 mag arcsec $^{-2}$).

structure ($m_R \approx 22.7$) about 5 arcsec from the centre (PA ≈ 240), possibly connected with a faint knot about 12 arcsec away from the nucleus. These features probably caused the elongation noted in previous lower-resolution optical images⁹⁻¹¹.

Both frames (Fig. 1*b*) of H1722 + 11, an X-ray source recently identified^{12,13} with a strongly polarized BL Lac, show the pres-

ence of a faint ($m_R = 20.6$), unresolved object, 2.6 arcsec southwest of the unresolved central image (Fig. 2*b*). A tentative redshift $z = 0.018$ was derived¹³ from a single absorption line attributed to the host galaxy. We note, however, that if the host galaxy is a giant elliptical, it should show up clearly in our CCD frames. Spectroscopic data obtained by one of us (R.F.) in

TABLE 2 BL Lac companions

Object	Underlying galaxy	Distance (arcsec)	Companion m_R	PA
PKS0048-09	—	(1.6)	(22.0)	(80)
H0323+02	detected*	2.3	21.6	230
H0414+00	detected	—	—	—
H1722+11	—	2.6	20.6	235
H2155-30	—	4.5	19.9	90
H2351-31	detected	—	—	—

Uncertain values are given in parenthesis.

* Host galaxy previously detected.

August 1989 failed to confirm the proposed redshift.

Around PKS2155-30, one of the brightest (a visual magnitude $m_V = 12.8-14.0$) and most studied BL Lac, the presence of an asymmetric nebulosity with a longer and wider extension to the east was reported¹⁴ ten years ago. Spectroscopy of the nebulosity through a 2-arcsec slit centred 4 arcsec to the east of the nucleus showed absorption features from a stellar population at $z = 0.117$ (ref. 15). We obtained two short exposures of the source near minimum brightness ($m_V = 13.8$). No evidence of nebulosity around PKS2155-30 (down to a surface brightness of $\mu_R = 23 \text{ mag arcsec}^{-2}$) is present, but a relatively faint ($m_R \approx 20$) object, ~ 4.5 arcsec east of the nucleus (in the direction of the previously claimed asymmetry), is apparent in both images (Fig. 1c). This object appears to be resolved, with some elongation in the east-west direction. At the hour angle of this observation the absence of fine guiding resulted in a point spread function that was elongated east-west and this may be responsible, in part, for the elongation of the image. According to the slit position quoted¹⁵, the stellar absorption lines were detected in spectra of a region centred almost exactly on the newly resolved object: the redshift $z = 0.117$ could therefore refer to it, rather than to PKS2155-304 itself.

A somewhat ambiguous case is presented by PKS0048-09, a bright rapidly variable BL Lac for which neither a redshift nor indication of surrounding nebulosity has been previously reported. Our frame shows an asymmetric elongation extending 2.5 arcsec to the east at a level of $\mu_R = 22.2 \text{ mag arcsec}^{-2}$ (Fig. 1d). This elongation is also present in the 10-s exposure to white light. This can be either ascribed to the presence of an asymmetric nebulosity surrounding the BL Lac to a close (≈ 1.6 arcsec) faint ($m_R \approx 22.0$) object.

For two other objects, H0414+00 and H2351-31, we detect the presence of an elliptical nebulosity that has not been previously reported. In H0414+00 a bright point-like source is surrounded by a nebulosity (ellipse ratio, $b/a, \sim 0.8$; PA of major axis = 70) extending out to 3.5 arcsec at surface brightness $\mu_R = 23.5 \text{ mag arcsec}^{-2}$. In H2351-31 the elliptical nebulosity ($b/a \approx 0.65$; PA = 90) is more conspicuous and extends to 7.4 arcsec from the nucleus at $\mu_R = 24.2 \text{ mag arcsec}^{-2}$. Spectra of this nebulosity reveal absorption features of a stellar population at $z \approx 0.17$ (ref. 16).

Finally, for PKS0118-27 and PKS0138-09 no evidence of condensations or extended images (after accounting for the elongation of the point spread function) are found at the limit of $\mu_R = 22.5 \text{ mag arcsec}^{-2}$. For PKS0118-27, a relatively bright BL Lac ($m_V \approx 15.7$), an absorption redshift $z = 0.56$ was recently proposed¹⁷ on the basis of a single absorption feature attributed to Mg II ($\lambda = 2,800 \text{ \AA}$). No redshift is available for PKS0138-09. A substantial fraction of the BL Lacs observed have angularly close objects (Table 2). Owing to the limited number and arbitrary selection of the objects, however, we cannot assess the statistical significance of this.

The nature of the faint objects detected is unclear. Although the possibility that they are field stars and/or galaxies cannot be excluded, the probability P of chance juxtaposition of field stars and galaxies within 5 arcsec of a BL Lac is small. From the average counts of objects with $20.0 \leq m_R \leq 22.0$ in all our frames, we derive $P < 0.05$, consistent with that derived from the expected surface density of stars¹⁸ and galaxies¹⁹ in the magnitude interval considered. This is also consistent with the extrapolation of the counts of Webster *et al.*²⁰, taking into account the different magnitude interval considered and the presence of stars.

We therefore discuss the consequences of assuming that the faint objects detected at small angular distance from the BL Lacs are related to them. By analogy with the case of quasi-stellar objects (QSOs), we consider the possibilities that they are physically associated with the BL Lacs, or that they are due to gravitational lensing.

If physically associated, these objects could represent condensations in the surrounding nebulosity or galaxies involved in some kind of tidal interaction with the BL Lacs, as found in a large fraction of QSOs^{21,22}. The complex morphology of the structures around H0323+02 (Fig. 1a) is suggestive of the presence of large distortions or condensations of gas. But contrary to the case of QSOs, no extended line emission was found around this object²³. The object close to PKS2155-30 could be a nearby galaxy, as found in few other BL Lacs^{24,25}.

By analogy with QSOs, for which gravitational lensing has been reasonably demonstrated in a number of cases (see, for example, ref. 26), we propose an interpretation of our results in terms of gravitational lensing. Gravitational lensing by stars in a foreground galaxy (microlensing) has been proposed⁵ to explain the peculiar properties of the BL Lacs. Stars, in particular, would selectively amplify the continuous emission relative to lines and, in some cases, the galaxy as a whole could produce multiple images^{5,27,28}. This could be the case for H1722+11 and H0323+02, where angular separation of the images is close to that expected for lensing by a galaxy²⁸. We note, however, that no galaxy has yet been detected along the line of sight to H1722+11 and that the very complex structure of the galaxy around H0323+02 prevents us from verifying the off-centering of the BL Lac that the microlensing predicts⁵. Owing to the virtual absence of emission lines, gravitational lensing effects in BL Lacs may be more difficult to determine than in the case of QSOs. □

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Periodic features in Saturn's F ring: evidence for nearby moonlets

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THE Voyager 1 and 2 spacecraft^{1,2} found that the F ring of Saturn contains a remarkable diversity of features, from kinks and clumps to the so-called braids, with typical longitudinal spacings estimated to range from 5,000 to 13,000 km. Because any nearby satellite perturbing a narrow ring should produce periodic features of a single wavelength³⁻⁵, the F ring's 'shepherd' satellites, Prometheus and Pandora, have been suspected to cause the observed periodicities. To test this simple idea, we have now examined a selection of the best available Voyager images of Saturn's F ring by applying a fast Fourier transform technique to azimuthal profiles from spacecraft ring images. Only a few distinct periodic signals, including one due to the inner shepherd, are visible. We suggest that these periodic signatures provide evidence for so-far-undiscovered satellites next to this puzzling ring.

When a satellite interacts gravitationally with a nearby narrow ring, the phasing of the perturbations felt by each ring particle introduces a wave downstream of the moon, even when all objects start on circular orbits. The nominal wavelength λ is $3\pi\Delta a$, where Δa is the separation in semi-major axes of the ring and the orbit of the perturbing moon; λ manifests the motion of ring particles relative to the perturber during one sidereal period. The radial amplitude of the wave is $\sim a^3/(\Delta a)^2(m/M)$, where m and M are the masses of the satellite and of the primary body in the system (which is Saturn for the F ring) and a is the semi-major axis of the ring. Thus, nearby satellites are the most effective perturbers and, as long as processes are linear, each satellite should generate a single characteristic wavelength; for the F ring, the wavelength owing to Prometheus is 7,850 km and that owing to Pandora is 14,500 km. If the orbit of either the ring or the moon is elliptical, λ will remain $3\pi\Delta a$, as in the circular case. In addition the ring will become azimuthally clumped, initially having a spatial period of $3\pi\Delta a$, because the varying distance of the perturber from any ring particle at its closest approach^{5,6} induces different perturbation amplitudes.

To study the actual structure of a ring perturbed by a satellite, we analysed the variable brightness of the F ring by assembling two data sets, a composite of selected Voyager 1 images and a pair of overlapping Voyager 2 images. The former consists of 13 consecutive (Flight Data System (FDS) 35044.24–35045.36, average phase angle $\approx 120^\circ$) clear-filter ($\sim 4,800 \text{ \AA}$) narrow-angle images, each separated by 4.8 min with an average resolution of 40 km per pixel. These are the only high-resolution contiguous images that cover a substantial azimuthal fraction of the ring. The second set is a pair of Voyager 2 narrow-angle clear-filter pictures (FDS 43785.17 and 43805.50, having phase angles of $\sim 9^\circ$) with an average resolution of 90 km per pixel. Unfortunately, no Voyager 2 sequence has consecutive high-resolution images of overlapping ring arcs taken in quick succession. All images were calibrated and corrected for background light variations.

We calculated the radially integrated brightness or equivalent width of the F ring, $w = \int I/F dr$, where I is the absolute intensity, πF is the incident solar flux density and r is the radial distance from Saturn's centre. As the resolution and motion-

induced smear of individual images vary considerably, we used w instead of local brightness, because w is a smear- and resolution-independent quantity. The equivalent width was calculated as a function of orbital longitude θ for each image and then the longitudes were translated to a common time, allowing the data sets to be spliced together into a larger mosaic. A further background subtraction was performed by removing the average I/F along nearby arcs contiguous to the visible ring.

Figure 1 shows w plotted against θ for the two data sets described above. Figure 1a is the clear-filter sequence shown over an azimuthal coverage of $\sim 150^\circ$ (with a resolution of 0.1°). The data set contains two very narrow peaks due to bright clumps, which can be tracked from image to image as they move with the mean motion of the F ring. Such anomalous brightenings may indicate the presence of embedded moonlets^{2,7} but, because of insufficient image resolution, we cannot distinguish such bodies from local density enhancements of small particles. These brightness knots may be related to the unknown physics of Neptune's ring arcs⁸. Besides the brightness clumps, other non-periodic structure is also visible in Fig. 1a.

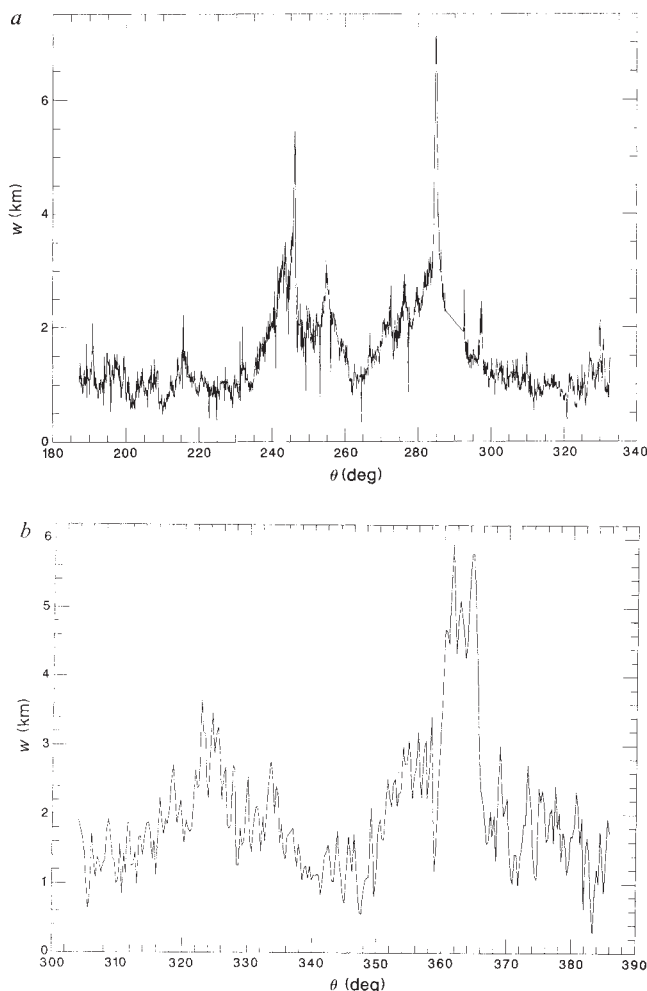


FIG. 1 a, Composite plot of equivalent width w as a function of longitude for the Voyager 1 narrow-angle clear-filter mosaic. All images have been translated to the time of the initial image in the sequence (35044.24), taken at Julian date 2,444,559.8365. Prometheus and Pandora have longitudes of 136.6° and 9.1° , respectively, at this time. The longitude data are given in a reference frame where 0° is located at the ascending node of Saturn's equatorial plane as it crosses Earth's equatorial plane, measured from the first point of Aries in AD 1950.0. b, Similar plot for the Voyager 2 composite image (43785.17 and 43805.50) translated to the time of the first image (Julian date 2,444,835.2972), Prometheus and Pandora have longitudes of 271.6° and 228.6° in this plot.

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TABLE 1 Voyager 1 data

Feature	k (deg ⁻¹)	λ_θ	$360^\circ/\lambda_\theta$	λ_{km}	$\Delta a = \lambda_{km}/3\pi$
1	9.77×10^{-3}	102.4°	3.5	—	—
2	1.95×10^{-2}	51.2°	7.0	—	—
3	4.39×10^{-2}	22.8°	15.8	—	—
4	6.84×10^{-2}	14.6°	24.6	35,770	$3,800 \pm 400$
5	9.28×10^{-2}	10.8°	33.4	26,360	$2,800 \pm 210$
6	0.122	8.2°	43.9	20,050	$2,130 \pm 125$
7	0.220	4.5°	79.2	11,120	$1,180 \pm 40$
8	0.313	3.2°	112.5	7,820	830 ± 20

$360^\circ/\lambda_\theta$ is the azimuthal wavenumber and λ_{km} is the resulting wavelength, based on a nominal semi-major axis for the F ring, 140,180 km. The last column lists the separation distance in km and its estimated error if a perturbing satellite caused such a feature (compare with refs 3–5).

The data were interpolated and re-binned for application of the fast Fourier transform (FFT). Using standard algorithms⁹, we evaluated the power as a function of wave number k ($1/\lambda_\theta$, where λ_θ is the azimuthal wavelength in degrees). Results from the FFT calculation are displayed in Fig. 2a; because significant power was not visible at very short wavelengths, only the large-wavelength end of the spectrum is shown, although we calculated values up to the Nyquist frequency. We have performed numerous checks of the errors and uncertainties associated with the transform procedure¹⁰ and concluded that the reported features are robust. The transform results are obviously dominated by the spacing between the two clumps. Once this power is removed, confidence intervals can be calculated for the other features.

The few specific wavelengths containing significant power in the Voyager 1 data are listed in Table 1 along with relevant information; our discrete number of features is in sharp contrast to preliminary post-encounter Voyager reports². It is clear that Prometheus gives rise to λ_8^1 (superscript 1 indicates Voyager 1) because the calculated Δa matches that expected for the satellite's orbital spacing ($\Delta a = 832 \pm 30$ km, ref. 11) and because this feature stands markedly above the noise in both data sets. There is, however, no signal from Pandora ($\Delta a = 1,520 \pm 30$ km, ref. 11)—the perturbation amplitude estimated above predicts that Pandora's signal would be only $\sim 10\%$ as strong because Pandora is both less massive and farther from the ring than Prometheus.

We suggest that λ_7^1 is produced by a moonlet lying at $\Delta a = 1,180$ km, even though Voyager would have easily detected an object massive enough to induce this wave if that object were on a circular orbit at the inferred separation. A moonlet well below the Voyager detection limit¹² ($r \geq 10$ km) on an eccentric orbit ($e \approx 10^{-3}$), can pass the ring close enough to disturb it appreciably⁵, spawning an evanescent wave somewhat downstream from its point of closest approach. To explain charged-particle absorptions measured by Pioneer 11, Cuzzi and Burns¹³ have proposed that a swarm of moonlets lie about the F ring. Such objects would develop slightly eccentric and inclined orbits (i and $e \approx v_e/v_c \approx 10^{-3}$, where v_e and v_c are the surface escape

velocity and the orbital velocity of typical objects) by gravitational interactions with the shepherds and with one another¹⁴.

Waves of different frequencies may beat with one another to produce a signal. This is probably the origin of λ_5^1 and λ_6^1 because $k_5^1 \approx k_8^1 - k_7^1$ and $k_6^1 \approx 2k_7^1 - k_8^1$. Furthermore, by numerically removing the two large peaks in the data, we have demonstrated¹⁰ that λ_1^1 , λ_2^1 and λ_3^1 , which result from their influence, should be ignored as they do not represent an induced periodicity.

Wavelength λ_4^1 is more troublesome. It is difficult to argue that this signal is caused by a small moon ($r \approx 10$ km) on an eccentric orbit with $\Delta a \approx 4,000$ km because the eccentricity would have to be $\sim 10^{-2}$, about an order of magnitude larger than expected to arise from gravitational stirring, even by the shepherds. Moreover, such a perturber could not be on an eccentric orbit interior to the ring without colliding with the main ring system. If the responsible satellite were on a circular orbit interior to the ring, the deduced Δa would place it near the outer edge of A ring, a position where such an object should have a profound influence that is not observed. A satellite embedded in the ring proper can also produce periodic features^{10,15–17} but it is unclear how such a perturber could generate long-wavelength signals. The beat features $k_8^1 - k_4^1 = 0.245$ and $k_7^1 - k_4^1 = 0.152$ are marginally significant in Fig. 2a.

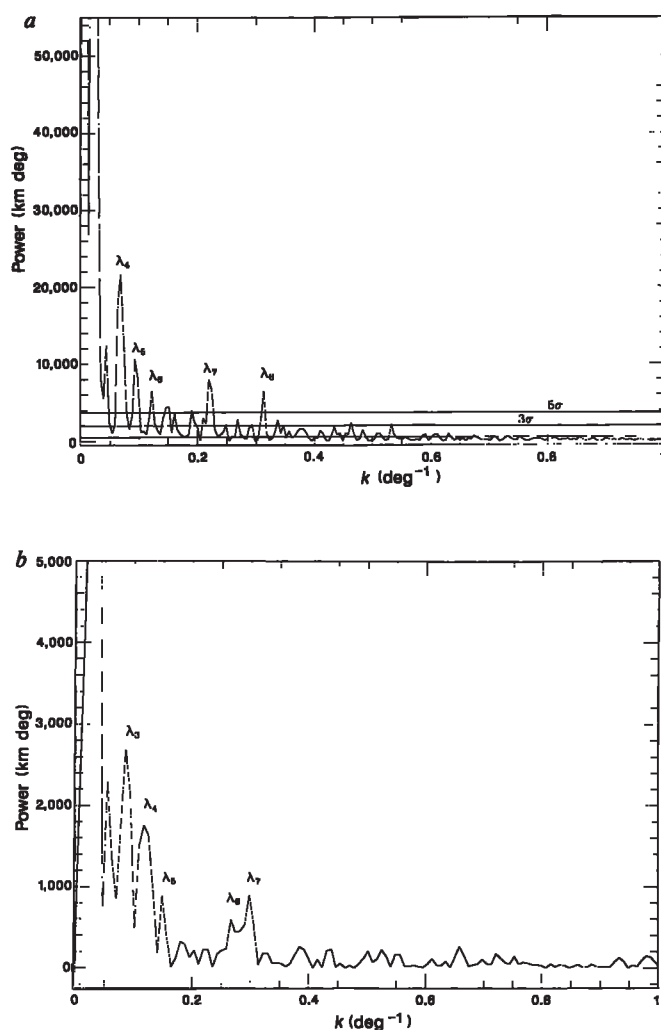


FIG. 2 a, Power spectrum for the Voyager 1 data in Fig. 1a as a function of wavenumber k . The horizontal lines represent the mean (with the long-wavelength power removed), 97% (3σ) and 99.9% (5σ) confidence intervals. b, Power spectrum for the Voyager 2 data in Fig. 1b.

TABLE 2 Voyager 2 data

Feature	k (deg ⁻¹)	λ_θ	$360^\circ/\lambda_\theta$	λ_{km}	$\Delta a = \lambda_{km}/3\pi$
1	3.13×10^{-2}	31.9°	11.3	—	—
2	5.49×10^{-2}	18.2°	19.8	44,560	$4,730 \pm 1,070$
3	8.62×10^{-2}	11.6°	31.0	28,380	$3,010 \pm 400$
4	0.117	8.5°	42.1	20,910	$2,220 \pm 200$
5	0.149	6.7°	53.6	16,420	$1,740 \pm 135$
6	0.267	3.7°	96.1	9,160	970 ± 40
7	0.298	3.4°	107.3	8,210	870 ± 35

Owing to its much more limited azimuthal coverage, our Voyager 2 data set (Fig. 1b) yields much poorer spectral resolution. This accounts for the cruder relative errors in separation (Table 2) and also means that any azimuthal wavelength longer than one-half the total longitude ($\lambda \geq 30^\circ$) is not reliably resolved and should be ignored. Despite these limitations, the signal of Prometheus (λ_2^2 , where the superscript indicates Voyager 2) seems to be visible and beating relations may be present, $k_4^2 \approx k_6^2 - k_3^2$ and $k_2^2 \approx k_3^2 - k_1^2$. The various wavelengths identified in Table 2 and seen in Fig. 2b may indicate the presence of other satellites as discussed in the case of λ_1^1 . The quality of these data is insufficient to allow unequivocal statements, however, and they are presented primarily for the sake of completeness because they comprise the best available information from Voyager 2.

We are intrigued that wavelength λ_3^2 lies within the error bars of λ_1^1 , perhaps implying some continuity in the ring over an extended period of time. If ring features are not being continually re-imprinted, their longevity will depend on the nature of damping in dense narrow rings, a poorly understood topic at present¹⁸. To see the effects of damping and the role of the relative positions of the perturbers, we have analysed subsets of the Voyager 1 data; our results once again are mixed, partly because of the low resolution of the data.

To unravel the genesis of the F ring's puzzling features, one must completely describe all perturbers in this region. Such information may also help identify the processes that occur in

regions near the Roche limit (where large bodies may be disrupted by local tidal forces), as well as the size distribution of ring particles. Both of these issues are crucial when trying to apply our understanding of the physics of planetary rings to the primordial solar nebula. The planned Cassini Orbiter should provide the temporal and spatial resolution needed to solve the puzzles of the F ring. □

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A fuel cell for the partial oxidation of cyclohexane and aromatics at ambient temperatures

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THE great interest in the catalytic partial oxidation of alkanes and aromatic hydrocarbons is motivated by a need to find new industrial routes for processing these hydrocarbons, available from natural fuels, selectively and in one step under mild conditions^{1–5}. Here we report the partial oxidation of saturated and aromatic hydrocarbons at the cathode of an O_2/H_2 fuel cell at ambient temperatures. With a cathode comprising a mixture of alkaline-earth or rare-earth metal chlorides and graphite, catalytic synthesis of cyclohexanol and cyclohexanone from cyclohexane was effected with 100% selectivity. Among the catalysts tested, $SmCl_3$ /graphite was the most active. The same fuel-cell system is effective also for hydroxylation of benzene and toluene. The mechanism may involve generation of active oxygen on the cathode by the normal O_2/H_2 fuel-cell reaction.

The simultaneous generation of electricity and useful chemicals in fuel cells has been demonstrated^{6–10}. Despite the expectation that alcohols, aldehydes and ketones could be synthesized using hydrocarbons as fuels at the anode, neither partial oxidation of alkanes nor hydroxylation of aromatic hydrocarbons has yet been achieved. When we used alkanes or aromatic hydrocarbons as fuels at the anode, no oxidation reactions occurred at ambient temperatures. Although the deep oxidation product was observed. When cyclohexane or benzene were introduced on the cathode side of an O_2/H_2 fuel cell, however, partial oxidation reactions did occur.

In our fuel cell (Fig. 1) a silica-wool disk, holding 0.25 ml of 1 M H_3PO_4 , separates the anode and cathode compartments. The cathode was prepared from a mixture of the metal chlorides

(0.5 mol%) in a powder of graphite (70 mg) and teflon (5 mg). This mixture was pressed to a wafer on a hot plate at 393 K. The anode was prepared by a similar method except that 20 mg of platinum black were used instead of metal chloride. The electrodes were 3.8 cm² in area and 0.1 mm thick. Oxygen (101 kPa, flow rate of 5 ml min⁻¹) was bubbled through the cyclohexane, benzene or toluene (40 ml) in the cathode compartment. Hydrogen (98 kPa) and water vapour (3.3 kPa) were passed (10 ml min⁻¹) through the anode compartment. The reaction, initiated by short-circuiting the cell, was allowed to proceed for 20 h at 298 K. The products were analysed by gas chromatography.

Using a graphite cathode containing no metal chloride, only a small amount of cyclohexanol and cyclohexanone was formed

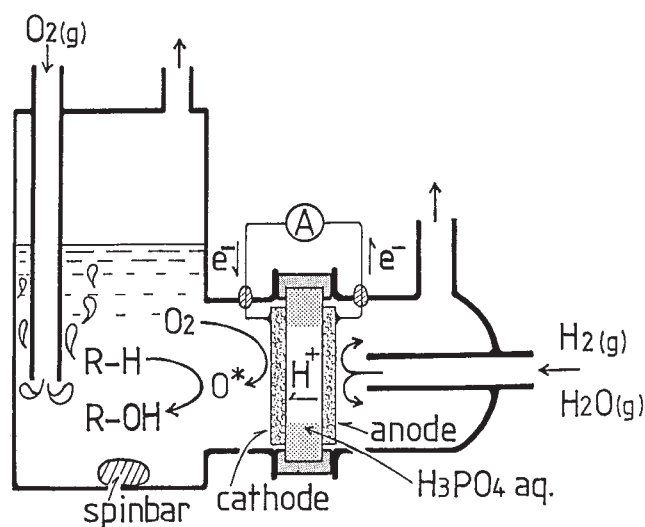


FIG. 1 Schematic diagram of the O_2/H_2 fuel cell for the partial oxidations of alkanes and aromatics.

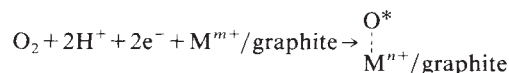
from cyclohexane under short-circuit conditions. The addition of alkaline-earth and rare-earth metal chlorides to the graphite increased both the current and the product yield. The catalytic activities of cathodes containing metal chloride improved with repeated use as long as the electrolyte was renewed before each reaction. The results obtained for the second usage of cathodes containing various metal chlorides are shown in Fig. 2. Figure 2 shows that the catalytic activities of the alkaline-earth and rare-earth metal chlorides are greater than that of FeCl_3 . The current efficiencies for the sum of the two products are 4–7% for most of the electrodes tested. Here, the current efficiencies were calculated assuming that the cyclohexanol and cyclohexanone are formed independently through the two- and four-electron oxidations, respectively. The rest of the current is ascribed to the formation of water in the O_2/H_2 fuel-cell reaction. The turnover numbers (number of molecules reacted per active site) for Y^{3+} (1.01), La^{3+} (1.01), Pr^{3+} (1.13), Nd^{3+} (1.62), Sm^{3+} (1.71), Eu^{3+} (1.02), Dy^{3+} (1.00), Ho^{3+} (1.00) and Lu^{3+} (1.06) exceed unity. This was also the case for the other rare-earth metal cations and for the Mg^{2+} and Ca^{2+} when the reaction was carried out for a further 20 h. The integrated turnover number for the Sm^{3+} exceeded 10.0 after six successive reactions. It is obvious that most of the cations in Fig. 2 work as electrocatalysts. For the partial oxidation of cyclohexane, Sm^{3+} shows the highest yields for both products and the largest turnover number (1.71). We have therefore used a SmCl_3 -graphite cathode to study the reactions further.

Under open-circuit conditions at 298 K, the bubbling of a mixture of oxygen (50 kPa) and hydrogen (25 kPa) through the cyclohexane did not result in any oxidation. Under short-circuit conditions, when helium replaced hydrogen in the anode compartment, no oxidation occurred. When oxygen, but no hydrogen, was passed through the anode compartment, neither current nor oxidation products were observed. This shows that the anodic oxidation of cyclohexane as a fuel does not take place at room temperatures. The oxidation of cyclohexane occurs only when the cyclohexane is present at the cathode during the O_2/H_2 fuel-cell reaction.

The oxidations of benzene and toluene at a SmCl_3 /graphite cathode have been carried out under the same experimental conditions as those for the oxidation of cyclohexane except that the reaction time was only 3 h. Phenol (6.2 μmol , current efficiency 5.5%) is the only product from benzene. The oxidation

products of toluene are cresols (0.23 μmol , *o*-cresol:*m*-cresol:*p*-cresol = 0.13:0.02:0.08), and the side-chain oxidation products benzaldehyde (0.27 μmol) and benzylalcohol (0.07 μmol). We have confirmed that the oxidations of benzene and toluene occur only during the O_2/H_2 fuel-cell reaction.

The active oxygen species generated at the metal cations in the cathode may be responsible for the activation of the C—H bond in cyclohexane and aromatic hydrocarbons



where O^* could be HO^* , O_2^{2-} , HO_2^- or an oxo species^{11,12}. The large difference in the yield of product for the various metal cations in Fig. 2 suggests that the generation of the O^* is highly dependent on the metal cation. Repeated runs of the reaction may stabilize or increase the active cation sites in the graphite, resulting in the improvement of the catalytic activities of the electrodes. □

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A diagnostic for denitrification in the winter polar stratospheres

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A STRIKING negative correlation between *in situ* measurements of reactive nitrogen (NO_y) and nitrous oxide (N_2O) has been observed throughout the lower polar stratospheres^{1,2}. This correlation has been extensively used to determine reference values for NO_y from a measurement of N_2O and, hence, to quantify the extent of denitrification in high-latitude air parcels^{1,3}. (Denitrification in the atmosphere is defined as the permanent removal of reactive nitrogen.) The removal of NO_y from the Antarctic winter stratosphere maintains high concentrations of reactive chlorine, thereby priming the atmosphere for catalytic ozone destruction⁴. Here we present the pairwise correlation of the NO_y and N_2O data from the Southern and Northern Hemispheres. Both datasets show a linear correlation region, defined as a reference state, and regions of denitrification, where the correlation breaks down. Using two-dimensional photochemical model simulations of the atmosphere, we find a similar linear correlation between NO_y and N_2O , thereby establishing a theoretic-

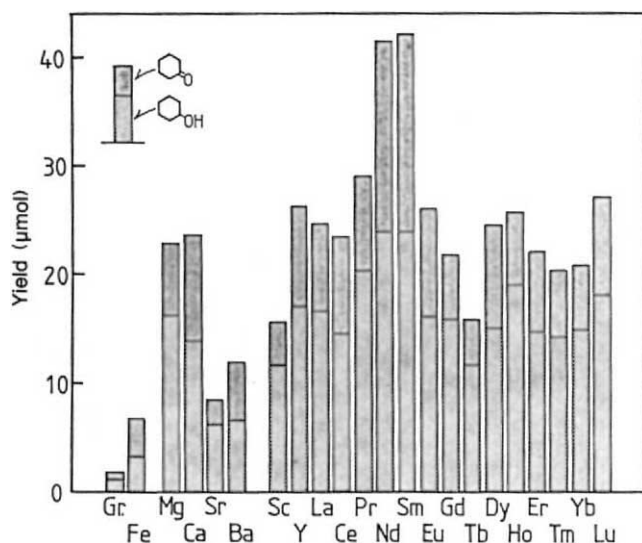


FIG. 2 Product yield in the oxidations of cyclohexane for the cathodes containing various metal chlorides.

cal framework for the reference state. This general approach, which can be extended to other pairs of molecules, should prove to be powerful in further comparisons of aircraft data with numerical models.

These *in situ* aircraft measurements of NO_y and N_2O in the stratosphere were obtained poleward of 53° during late winter in both hemispheres as part of NASA missions to study ozone depletion^{5,6}. Figure 1a shows data from the lower Arctic stratosphere from one leg of a flight on 12 January 1989. The scatter plot of these data in Fig. 1b shows the negative correlation which has been found to exist over a wide range of values. Poleward of 62°N the potential temperature along the flight track was $460 \pm 10\text{ K}$ corresponding to geometric altitudes near 18–19 km. Horizontal gradients in N_2O and NO_y at constant potential temperature are a general feature of the data in both missions (see, for example, refs 2 and 7). Overall, mixing ratio isopleths (contour lines) of N_2O and NO_y exhibit a steeper poleward slope than the isentropes (surfaces of constant potential temperature) because of the diabatic descent of air, which increases poleward^{8–10}. The large opposite vertical gradients of these species therefore create similarly opposed, horizontal gradients along the flight track. The boundary of the polar vortex along the flight track, located from the wind data, is at about

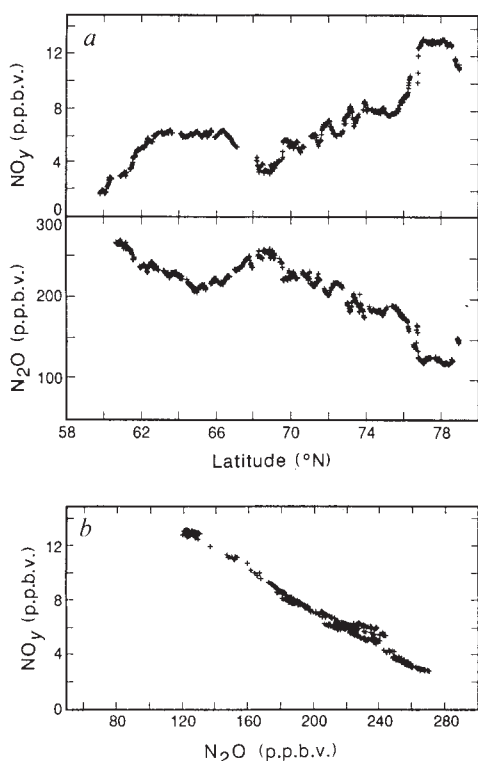


FIG. 1 a, NO_y and N_2O data plotted as 10-s average mixing ratios from the northbound leg of the flight on 12 January 1989 in the lower Arctic stratosphere. The data were acquired at potential temperatures of $460 \pm 10\text{ K}$. Potential temperature, θ , is defined as $\theta = [T_{\text{amb}}(P(\text{mbar})/1,000) - 0.2857]$ where T_{amb} is the ambient temperature and $P(\text{mbar})$ the ambient pressure in millibar. Potential temperature is chosen as the vertical coordinate because air motion at constant θ is isentropic. The boundary of the polar vortex, defined by the maximum horizontal wind and the gradient in potential vorticity, is located at 74°N (ref. 11). N_2O is measured *in situ* with a second-harmonic absorption spectrometer operating near $1,300\text{ cm}^{-1}$ using a tunable diode laser source⁷. Measurements are recorded at 2 Hz, corresponding to 0.1 km of flight path, and are reported with an accuracy of $\pm 5\%$ (ref. 40). The NO_y measurement technique uses catalytic conversion of component NO_y species to NO on a surface of gold at 573 K with CO added as a reducing agent¹⁵. The NO product is subsequently detected in the chemiluminescent reaction with reagent O_3 . Measurements are recorded at 1 Hz and reported with an accuracy of $\pm 15\%$. b, NO_y - N_2O scatter plot of the data in a.

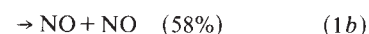
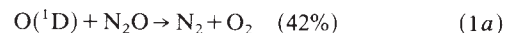
74°N near a mixing ratio of 180 parts per 10^9 by volume (p.p.b.v.) N_2O (ref. 11). A well defined polar vortex is a meteorological feature of the stratosphere in both winter hemispheres. Inside the vortex boundary during winter, the photochemistry of trace species is greatly perturbed from lower-latitude conditions, leading to ozone depletion^{12,13}. The small-scale horizontal gradients in these and other tracers are general features of the data near the boundary of both vortices (see, for example, ref. 9).

A summary of the data from several potential temperature levels for both missions is shown in Fig. 2. Both data sets show a distinct region of negative correlation above N_2O values in the range 160–180 p.p.b.v. These values are generally found outside the vortex boundary in air parcels that are largely chemically unperturbed^{2,14}. In the Arctic data, the correlation is observed with N_2O values as low as 100 p.p.b.v. in the earliest flights. To aid the discussion of a reference state, the average data are fitted to a straight line in the linear correlation region (Fig. 2b). The slope of the line relates the loss of N_2O to the production of NO_y for many air parcels sampled in similar physical locations. The values are 7% and 6.5% for the Antarctic and Arctic, respectively. In the linear portion of each data set, all potential temperature surfaces are well represented by the same linear-fit parameters. This suggests a constant relation over a wide altitude (16–20 km) and latitude (55° – 75°) range.

The negative, nearly linear correlation shown in Fig. 2b is assumed to be the unperturbed relation or reference state between NO_y and N_2O for N_2O values above ~ 100 p.p.b.v. At N_2O values below 160 p.p.b.v., many of the data points from both hemispheres fall significantly below the nominal 7% slope. The NO_y values for these data points are considered to be perturbed from normal values, as a result of denitrification in the associated air parcels. The difference between the observed NO_y and the reference value is therefore a measure of the NO_y removed from the air parcel^{1,15}. Denitrification and dehydration are believed to occur at low temperatures when polar stratospheric cloud particles composed of co-condensed H_2O and HNO_3 form and then settle^{15–17}. Because HNO_3 is the largest NO_y component in the unperturbed stratosphere, the amount of denitrification can be substantial¹. Independent evidence for the seasonal removal of condensable species from the lower polar stratosphere in winter comes from satellite observations of polar stratospheric clouds^{18,19}. Other inferences of denitrification from *in situ* measurements have relied only on relative changes in NO_y species between adjacent air parcels^{20,21}. The systematic differences in denitrification and dehydration between hemispheres are presented elsewhere²².

Other data can be re-examined to look for a correlation in regions in which local denitrification is not expected. Balloon measurements at 34°S at two altitudes fit a negative correlation above 160 p.p.b.v. N_2O (ref. 23). In this case, the line (Fig. 2c) has a comparable slope, but a higher intercept. Furthermore, data from the Atmospheric Trace Molecule Spectroscopy (ATMOS) instrument on board Spacelab 3 were acquired remotely at mid-latitudes in May 1985 between 20 and 50 km (refs 24, 25). The scatter plot (Fig. 2c) of these data shows a negative correlation that is similar to that of the polar data in both slope and intercept above 100 p.p.b.v. N_2O .

The photochemistry of NO_y and N_2O in the stratosphere is strongly linked. The NO_y reservoir is mainly composed of NO and the higher oxides NO_2 , HNO_3 and N_2O_5 , which are formed by the oxidation of NO. The principal source of NO in the lower to middle stratosphere is N_2O according to the reaction^{26–28}



where $\text{O}(^1\text{D})$ is produced by photolysis of O_3 at wavelengths $< 310\text{ nm}$. NO_y mixing ratios are typically < 2 p.p.b.v. at the

tropopause, increasing at higher altitudes to maximum values of ~ 20 p.p.b.v. below 40 km (refs 11, 25, 29). Other sources of NO_y , such as lightning and cosmic-ray activity²⁶ are not considered important for the discussion here.

N_2O is up to several hundred times more abundant than NO_y throughout most of the lower atmosphere. Because of its low chemical reactivity and low photodissociation cross-sections, N_2O mixing ratios are largely constant throughout the troposphere, near 305 ± 2 p.p.b.v. (ref. 30). The primary loss of N_2O occurs in the stratosphere by ultraviolet photolysis with wavelengths < 230 nm



with a lesser contribution from reaction (1). As a result, mean N_2O values decrease monotonically with increasing altitude in the stratosphere³¹. The distribution of NO_y production in reaction (1b) and of N_2O photolysis in reaction (2) together form

the photochemical basis for the negative correlation between NO_y and N_2O in the lower stratosphere. Therefore, the slope of a linear correlation found in measurements over a wide range of N_2O values represents the effective conversion efficiency (ECE) of N_2O to NO_y in the associated air parcels. In the upper stratosphere (above 40 km), the correlation cannot be maintained because NO_y loss increases significantly owing to the reaction



with N produced principally by photolysis of NO .

To establish a theoretical framework for the correlation and the reference state, a model that includes radiation, chemistry and dynamics is essential. The use of a model provides insight into the balance between production of NO_y through reaction (1b) and loss of N_2O through reactions (1) and (2), and the degree to which photochemical destruction of NO_y through

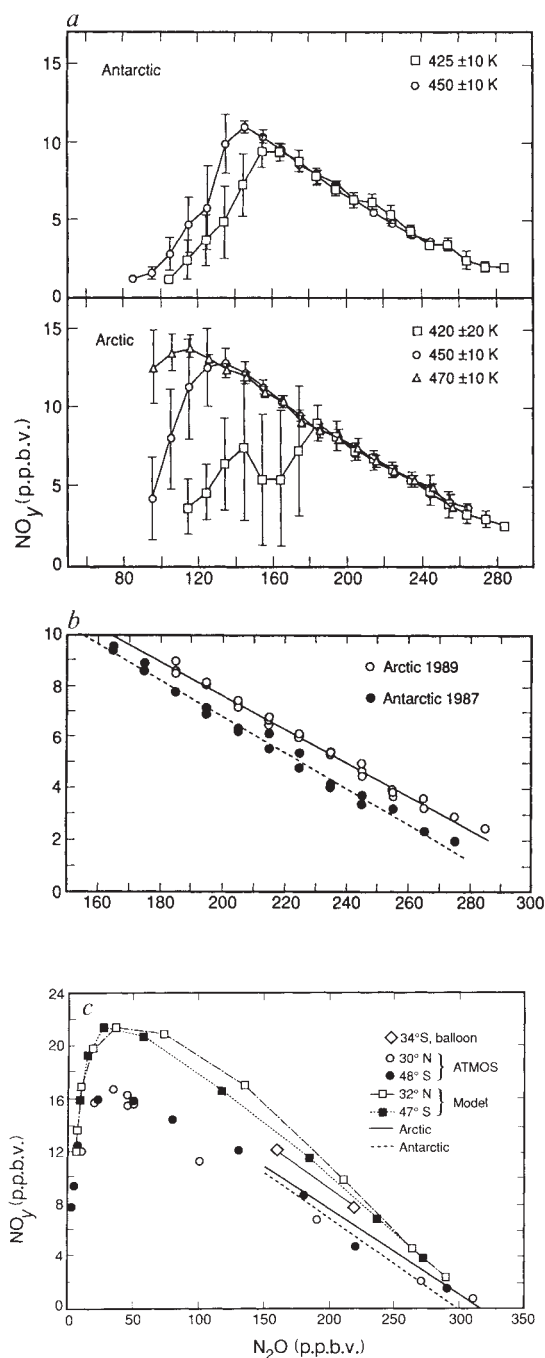


FIG. 2 Composite NO_y - N_2O scatter plots for the data acquired during the Airborne Antarctic Ozone Experiment (AAOE) based in Punta Arenas, Chile (53°S , 71°W) in August and September 1987 (ref. 5) and during the Airborne Arctic Stratospheric Expedition (AASE) based in Stavanger, Norway (59°N , 6°E) in January and February 1989 (ref. 6). The data are chosen to exclude measurements made when aerosol particles containing NO_y were present in the sampled air parcel¹⁵. The data are separated by potential temperature as calculated from observed pressure and temperature and grouped into 10-p.p.b.v. intervals of N_2O . Because temperatures decrease poleward at constant altitude along the flight track, potential temperature does not uniquely specify geometric altitude. The potential temperature range 400–480 K generally corresponds to the geometric altitude range 16–20 km, however, with an average proportionality constant of $18\text{--}22\text{ K km}^{-1}$ (ref. 8) (420 K, 17 km; 425 K, 17.3 km; 450 K, 18 km; 470 K, 19.5 km). Each symbol represents the average of the data in each interval with the vertical bars indicating the sample standard deviation. Each average represents between 100 and 2,000 1-s measurements. *a*, Data from seven Antarctic flights reaching 72°S (adapted from ref. 1) and from nine Arctic flights reaching 78°N on average¹¹. *b*, Data points from *a* that show a linear correlation. The slope of the linear least-squares fit to these points gives ECE values of 7% and 6.5% ($\text{NO}_y/\text{N}_2\text{O}$) for the Antarctic and Arctic, respectively, with a standard deviation of the fit of $\pm 2\%$. The uncertainty in the slopes arising from the uncertainty in the individual measurements is estimated to be $\pm 16\%$ (1σ) of the ECE values. *c*, Scatter plot of NO_y and N_2O data taken with the ATMOS instrument between 30 April and 6 May 1985 during the Spacelab 3 mission^{24,25}. Data points span 14–50 km geometric altitude in 4-km intervals with the absolute uncertainty for each measurement in the range of $\pm(10\text{--}20)\%$. The lines represent the linear fit to the Antarctic and Arctic data in *b*. The balloon data are from ref. 23. The squares are the results of the two-dimensional model simulation between 18 and 50 km for 30 April.

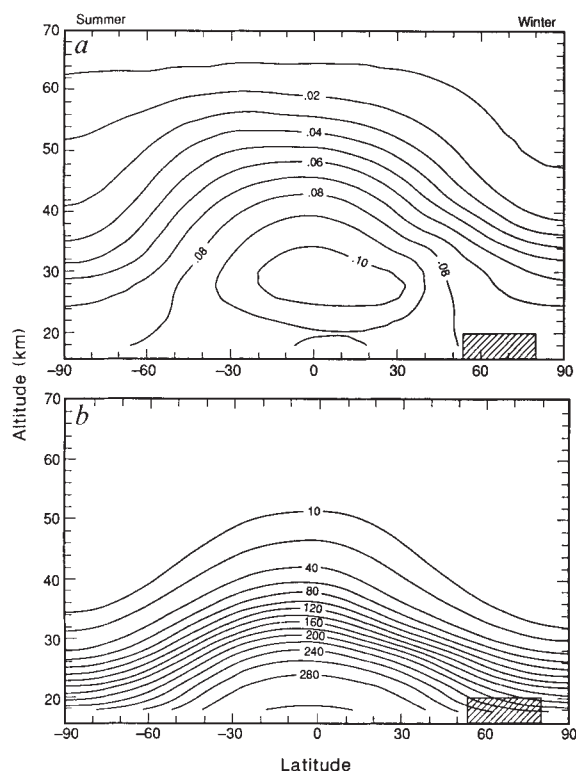
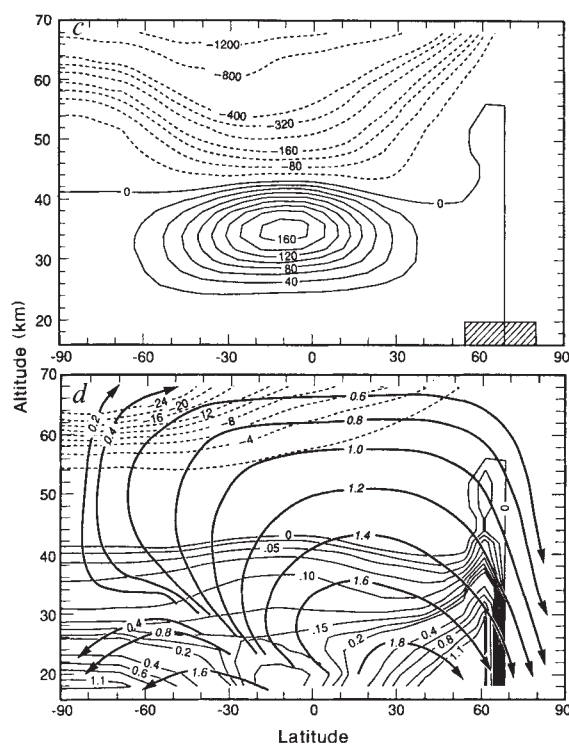


FIG. 3 Results from the two-dimensional model simulation of Garcia and Solomon³⁸ for 2 January corresponding to winter noon conditions in the Northern Hemisphere. The model is a time-dependent photochemical simulation of the atmosphere that produces zonally averaged tracer fields in the middle atmosphere by simulating the interaction of dynamics, radiation and chemistry. The model transport relies on the stream-function equation for the residual eulerian meridional circulation. Horizontal mixing of trace species is parameterized by an eddy mixing coefficient K_{yy} that is independent of latitude and season with a value of $3 \times 10^9 \text{ cm}^2 \text{ s}^{-1}$. The model results necessarily show a seasonal symmetry between the hemispheres because the model dynamics are assumed to be the same in both hemispheres. After defining initial conditions and boundary values, we allowed the model to reach a quasi-steady state, which requires several model years. The hatched area in the panels represents the approximate altitude and latitude



range of the observations in both hemispheres. *a*, Contours of effective conversion efficiency (ECE). The ratio is defined by $(NO_y(\text{p.p.b.v.}) - 1.5) / (300 - N_2O(\text{p.p.b.v.}))$ where 1.5 p.p.b.v. and 300 p.p.b.v. are the model tropical tropopause values assumed for NO_y and N_2O , respectively. *b*, Contours of N_2O mixing ratio. The contour between 10 p.p.b.v. and 40 p.p.b.v. corresponds to 20 p.p.b.v. *c*, Contours of the local instantaneous value at noon of the net production rate of NO_y , $NO_y(\text{production} - \text{loss})$, in units of $10^{-8} \text{ p.p.b.v. s}^{-1}$. The rates depend on the abundances of N_2O , O_3 and other species, rate coefficients of reactions (1)–(3), and radiation fields⁴¹. *d*, Contours of the local instantaneous conversion efficiency at noon of NO_y production from N_2O $[NO_y(\text{production} - \text{loss})]/[N_2O(\text{loss})]$, as calculated at each model grid point. The heavy solid lines show the meridional mass-stream function calculated for 2 January⁴². The contour values are the logarithm of the absolute value of the function in units of $\text{kg m}^{-1} \text{ s}^{-1}$.

reaction (3) also has a role. As the photochemical lifetimes of both NO_y and N_2O below 35–40 km are expected to exceed a year³², atmospheric mixing and circulation between the tropics and high latitudes are also relevant factors.

The two-dimensional model used here is described in Fig. 3. ECE values calculated at each model grid point are shown in Fig. 3a for 2 January, corresponding to Arctic mid-winter. Little change is found for 19 February, which is more appropriate for the late-winter observations in the Antarctic. Of most interest is the winter hemisphere, where a substantial region defined by altitudes below 28 km and latitudes greater than 50° contains ECE values between 6% and 8%, in excellent agreement with values observed there. In general, the gradients in the efficiency are small, both horizontally and vertically below 40 km, and the seasonal asymmetry is small. In the tropics and mid-latitudes, the altitude profile shows a broad maximum. The importance of the photochemical loss of NO_y in reaction (3) is demonstrated by the decrease in ECE values with altitude above 28 km to values $< 2\%$ in the upper stratosphere and mesosphere. A similar decrease occurs in the ATMOS data in Fig. 2c. The model values, especially at high latitudes, would be greater above ~ 40 km in winter if mesospheric and thermospheric sources of NO_y were included.

The agreement with model ECE values must be qualified by the underlying values of NO_y and N_2O . In general, the observed

NO_y and N_2O isopleths are considerably steeper and lower than the modelled isopleths throughout the measurement region. The model isopleth of N_2O for 160 p.p.b.v. shown in Fig. 3b is $\sim 4\text{--}5$ km higher than that from the Antarctic observations at 65°S , the average latitude of the vortex boundary³³. These, and similar discrepancies in the comparison with three-dimensional model results³⁴, highlight the difficulty in modelling high-latitude transport. The small vertical gradient of ECE in the observations and in the model poleward of 60° , however, reduce the importance of the discrepancy for this discussion. Specifically, Fig. 3b shows that the N_2O values in the range of observations vary from > 260 p.p.b.v. to ~ 160 p.p.b.v. These values span the range of the correlation in Fig. 2, but the distribution in latitude and altitude is quite different in the model. The area between the model ECE isopleths of 7% and 8%, poleward of 60° latitude, is considerably larger than the region of observation with N_2O values reaching 80 p.p.b.v. or lower. Curvature is found in the model correlation only for N_2O abundances < 50 p.p.b.v., consistent with the mid-latitude ATMOS data.

We also tested the effect of a lower horizontal eddy mixing coefficient on the model, in a manner similar to other studies^{35,36}. We found that the agreement with NO_y and N_2O could be significantly improved without appreciable changes in the basic linear correlation or the ECE values. A year-round change of this mixing parameter in the model is not warranted because

similar discrepancies in other values such as O_3 are not improved³⁶. Rather, the importance of this test is to show that the correlation and slope are robust features at high latitudes. The ATMOS data shown in Fig. 2c further suggest that large-scale, advective transport of air parcels containing >80 p.p.b.v. N_2O from mid-latitudes to the polar regions in the complete absence of horizontal mixing would still produce a linear correlation. These data are in fair agreement with the model, which clearly shows a linear correlation over a similar range of NO_y and N_2O values in Fig. 2c.

Evidence for the importance of photochemistry at low latitudes and poleward transport in creating the model ECE field is provided in Fig. 3c, d. In Fig. 3c, the net photochemical production rates of NO_y at noon are shown as an example of the spatial distribution of the conversion process. The rate maximizes in the tropics near 35 km and decreases to zero in polar night. The destruction rate for N_2O shows a similar pattern³⁷. The latitude and altitude dependence of the rate depends primarily on the distribution of solar ultraviolet. Model contours of the resulting NO_y mixing ratio show a similar symmetric distribution with a maximum near 23 p.p.b.v. between 35 and 40 km in the tropics. At the maximum NO_y production rate in the tropics, 20 p.p.b.v. of NO_y is formed in ~ 150 days, indicating that the timescale for transport into and out of this region must be of the same order.

By comparing the rates of net production of NO_y and destruction of N_2O , a local instantaneous conversion efficiency of N_2O to NO_y can be calculated at each model grid point. Between 28 and 41 km, the contours are nearly horizontal over a broad latitude range, with values $<15\%$ (Fig. 3d). Values in the region of maximum NO_y production are $10 \pm 2\%$, comparable to the 7% value observed at high latitudes. The largest instantaneous efficiencies are found at high latitudes where the maximum value of 116% corresponds to twice the branching ratio to NO in reaction (1). Observed ECE values are near $\sim 7\%$ and minimum net production rates indicate that high latitudes cannot be the region of N_2O conversion.

The link between model ECE values in Fig. 3a and the steady-state fields in Fig. 3c, d is transport and mixing. The general features of the zonally averaged transport are (see Fig. 3d): air rises in the tropics, moves poleward throughout the low and middle stratosphere, and descends at high latitudes^{31,38,39}. Horizontal and vertical eddy mixing are superimposed on this mean circulation. Horizontal mixing typically dominates vertical mixing in the lower stratosphere. Thus, transport of air occurs from the region of maximum photochemical production into the region of observation. Differences must exist between the instantaneous conversion efficiency and the observed ECE values, in part because of the residence time of air in the NO_y production region and mixing of air from the production region with air that has experienced greater NO_y loss at higher altitudes or less N_2O loss at lower altitudes. The linearity of the correlation over a wide range of N_2O values suggests that mixing must be effective. The long photochemical lifetimes of both species in the lower stratosphere imply, however, that mixing timescales need only exceed the timescale for descent from the NO_y loss region (N_2O values <50 p.p.b.v.) for a linear correlation to be maintained. Thus, the understanding of the model ECE field provided by Fig. 3 provides a high level of confidence in a linear correlation as a reference state for NO_y and N_2O . □

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Biological removal of dimethyl sulphide from sea water

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DIMETHYL sulphide (DMS) is an important sulphur-containing trace gas in the atmosphere. It is present in oceanic surface waters at concentrations sufficient to sustain a considerable net flux of DMS from the oceans to the atmosphere, estimated to comprise nearly half of the global biogenic input of sulphur to the atmosphere¹. DMS emitted from the oceans may be a precursor of tropospheric aerosols and of cloud condensation nuclei in the remote marine atmosphere, thereby affecting the Earth's radiative balance and thus its climate^{2–4}. Relatively little is known, however, about the biogeochemical and physical processes that control the concentration of DMS in sea water. Here we present data from incubation experiments, carried out at sea, which show that DMS is removed by microbial activity. In the eastern, tropical Pacific Ocean, DMS turnover is dominated by biological processes, with turnover times for biological DMS removal generally more than ten (3–430) times faster than turnover by ventilation to the atmosphere. Thus biological consumption of DMS seems to be a more important factor than atmospheric exchange in controlling DMS concentrations in the ocean, and hence its flux to the atmosphere. These results have significant implications for climate feedback models involving DMS emissions³, and highlight the importance of the microbial food web in oceanic DMS cycling.

We carried out experiments aboard the NOAA *Discoverer* as it sailed from 20° N to 23° S between 105° W and 110° W in February 1989. In freshly collected surface seawater samples,

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TABLE 1 Biological consumption and sea-air exchange of dimethylsulphide

Sample location	Depth (m)	Incubation temperature* (°C)	Dissolved DMS† (nM)	Biological consumption rate (nM d ⁻¹)	τ_{bio} (d)‡	DMS column burden (μmol m ⁻²)	Air-sea exchange rate (μmol m ⁻² d ⁻¹)	τ_{atmos} (d)‡	$\frac{\tau_{\text{atmos}}}{\tau_{\text{bio}}}$
20.5° N, 107.2° W	5	22	3.7	5.1	0.7	ND	—	—	—
14.8° N, 105.0° W	5	27	1.5	1.4	1.1	ND	—	—	—
10.5° N, 105.6° W	5	28	2.8	1.7	1.6	219	9.4	23	15
4.7° N, 108.5° W	5	26	10.3	18.0	0.6	266	24.0	11	12
1.1° S, 110° W	5	26	4.4	3.7	1.2	259	0.5	520	430
7.4° S, 108.3° W	5	26	2.4	1.1	2.2	306	6.0	51	23
15.3° S, 105° W	5	25	6.0	1.3	4.6	503	36.0	14	3
22.3° S, 105° W	40§	24	0.8	1.1	0.7	141	5.3	27	38

Biological turnover rates were determined by measuring the rate of DMS accumulation in the presence of chloroform and subtracting the rate of DMS accumulation in untreated samples. Duplicate bottles of each treatment were used. At least four time points over a 12-h period were used to calculate rates. Biological turnover times were calculated by dividing the measured DMS concentration in sample bottles by the DMS consumption rate in those samples. Turnover rates due to atmospheric exchange were calculated by dividing the column burden of DMS in the mixed layer, determined from depth profiles, by the estimated flux of DMS to the atmosphere⁴. ND, no data.

* Incubation temperatures were within 1°C of the *in situ* temperature.

† DMS concentrations measured in first reading of untreated samples; up to 2 h after collection.

‡ τ_{bio} is the biological turnover time and τ_{atmos} is the atmospheric turnover time.

§ Collected by hydrocast using Niskin bottle.

incubated with and without DMS additions, DMS concentrations increased for a time and then decreased (Fig. 1). The initial increases in DMS were probably due to the decomposition of endogenous dimethylsulphonioacetate (DMSP), which is believed to be the principal biogenic precursor of DMS in the ocean⁵⁻⁷. Both chloroform (an inhibitor of C_1 metabolism and possible general biocide) and a mixture of the broad-spectrum antibiotics—chloramphenicol and tetracycline (CAP-TET)—prevented the loss of DMS. These results indicate that DMS is biologically consumed by microbial metabolism in sea water.

Several possibilities exist for the aerobic metabolism of DMS, including use by methylotrophs and chemolithotrophs⁸⁻¹⁰. DMS could be also oxidized to dimethylsulphoxide (DMSO) by organisms as yet uncharacterized^{11,12}. Unfortunately, little or no information exists as to which of these processes are responsible for DMS removal in the sea.

Chloroform (CHCl_3) is a known inhibitor of C_1 metabolism¹³, therefore it is not surprising that it inhibited the consumption of DMS in sea water. In samples to which we added CHCl_3 , DMS always accumulated at a higher rate than in untreated samples (Fig. 2) because DMS consumption is inhibited by chloroform, whereas its production from dissolved and particulate DMSP is not affected by the inhibitor (R.P.K., manuscript in preparation). The increased rate of DMS accumulation in

the presence of chloroform therefore represents an estimate of its natural removal rate.

We have estimated DMS removal rates and biological turnover times for surface water samples collected in the eastern Pacific Ocean (Table 1). DMS consumption rates during incubations in the dark ranged from 1.1 nM d⁻¹ to 18.0 nM d⁻¹ yielding turnover times τ_{bio} ranging from 0.6 to 4.6 d. The high DMS concentration and consumption rate at 4.7° N were probably a result of increased productivity caused by nearby equatorial upwelling. DMS consumption rates were more strongly correlated with DMS concentrations ($r^2 = 0.741$; $n = 8$) than with particulate DMSP concentrations ($r^2 = 0.337$; $n = 7$).

Sea-air exchange of DMS, calculated from DMS column burdens, wind speed and sea surface temperature¹, ranged from 0.5 to 36 μmol m⁻² d⁻¹ for these sites, yielding turnover times τ_{atmos} ranging from 11.3 to 518 d for DMS in the mixed layer. The ratios of τ_{atmos} to τ_{bio} (surface samples only) ranged from a low of 3 at 15° S to a high of 430 near the Equator at 1° S. The exceptionally small contribution of atmospheric exchange to DMS cycling at 1° S was due to calm winds which resulted in very little surface ventilation.

Removal of DMS from sea water by mechanisms other than sea-air exchange has been suggested by several investigators^{5,14-17}, but no direct evidence has previously been

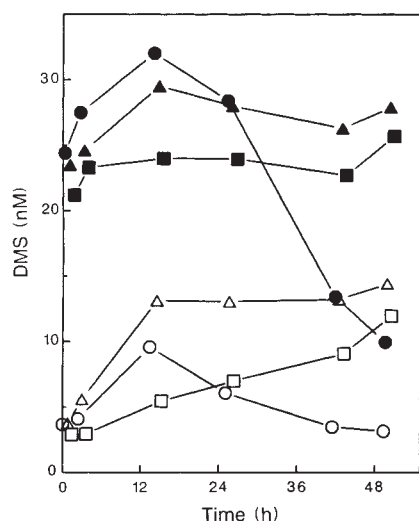


FIG. 1 DMS concentrations as a function of time in water samples incubated with or without spikes of DMS. Inhibitors were added to prevent microbial metabolism. Symbols: (○) no addition; (△) chloroform (500 μM); (□) antibiotics, chloramphenicol and tetracycline (125 mg l⁻¹ and 75 mg l⁻¹); (●) DMS; (▲) DMS + chloroform; (■) DMS + antibiotics. Data represent the means of duplicate bottles for each treatment and exhibited a range of ~10%. Water was collected while underway, from a pumping system located ~5 m under the bow of the ship. The station location was 20.5° N 107.2° W. Water samples (130 ml) were incubated in 140-ml silanized glass, acid-rinsed serum bottles sealed with teflon-faced septa and held in the dark at *in situ* temperature (22 °C). Subsamples for DMS analysis were taken periodically by removing the seal and taking up 5 ml with a teflon tube attached to a glass syringe. The water sample was then gently passed through a glass-fibre filter (Whatman GF/F) and introduced into a sparging system where the DMS was stripped from solution, and concentrated on a trap immersed in liquid argon. The cold-trap was replaced by hot water and the sulphur gases introduced into a gas chromatograph equipped with a Carboxen BHT 100 (2 m long, 1/8" diameter teflon) column and flame-photometric detector. DMS standards were prepared in ethylene glycol and a ship-board intercalibration with the NOAA/PMEL system² yielded agreement within 10%.

presented for biological consumption of DMS in natural ocean water. Our data indicate that, in most cases, biological removal of DMS in the surface ocean is much more important than DMS removal by ventilation. Even if our estimates of biological consumption are too large by a factor of 10, this process would still dominate over atmospheric exchange in all but one case (15 °S).

Other potential sinks for DMS in surface sea water (Fig. 3) include photochemical oxidation¹⁶ and particle adsorption with subsequent vertical transport to the deep sea¹⁷. Both of these processes are poorly understood. The only estimates that exist for these processes^{16,17} indicate that they are less significant on a global scale than atmospheric exchange of DMS, and therefore would yield longer turnover times than biological reactions. Because our data set is small, and covers only tropical and subtropical waters, further research is needed to compare the relative importance of biological consumption to other DMS sinks.

The fact that DMS is cycled quickly in sea water by biological reactions raises interesting questions regarding the possible role of DMS in climate feedback mechanisms. Predicting how DMS flux will change as a result of climate change, however, is a difficult task. The primary factors that control DMS fluxes are the sea-air transfer coefficient and the DMS concentration in surface water¹. The sea-air transfer coefficient is dependent on physical factors such as temperature and wind speed whereas the DMS concentration is a function of physical and biological processes related to its sources and sinks (Fig. 3).

Gross DMS production is linked to the production of its biogenic precursor DMSP which is produced by a subset of the algal population^{6,7,18}. Climatic controls on the phytoplankton, expressed through physical factors such as temperature, light and nutrient regimes, would be expected to influence the structure of the algal population (the biomass, productivity, species composition and size distribution, for example), as well as the total DMSP production by this population. Although it seems that DMS concentration is related to total DMSP (dissolved and particulate)^{6,7}, there are insufficient data to extend this conclusion to all ocean environments. DMS concentrations may not necessarily follow the total DMSP content of sea water if

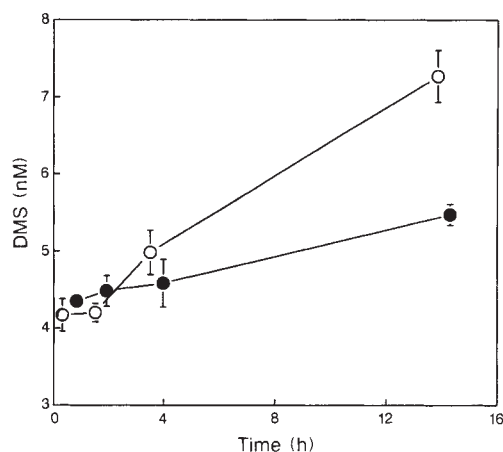


FIG. 2 Effect of chloroform (500 µM) on the concentration of DMS in sea-water samples incubated in the dark at *in situ* temperature (25 °C). Symbols: (●) untreated; (○) added chloroform. Data points represent the mean DMS concentration for three separate bottles. Vertical bars indicate one standard deviation. Higher or lower concentrations of chloroform produced a smaller stimulation (data not shown). The stimulation of DMS accumulation by chloroform varied in samples collected from different depths but was not significantly correlated with particulate DMSP. In separate experiments, chloroform did not significantly affect bacterial numbers or the disappearance of particulate or dissolved DMSP during 24-h dark incubations. The station location was 1.1° S 110° W.

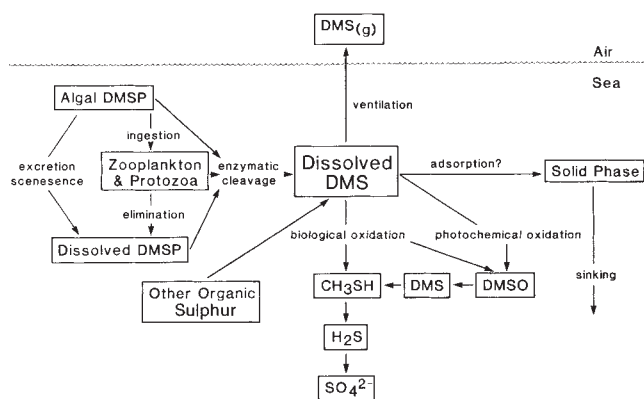


FIG. 3 Diagram illustrating the known sources and sinks for DMS in the surface ocean. Processes involving DMS production from algal DMSP are based on refs 15 and 19. The potential adsorption and photochemical destruction of DMS are based on the work of Brimblecombe and Shooter¹⁶ and Shooter and Brimblecombe¹⁷. The possible contribution of other organic sulphur compounds to DMS production is based on work by Kiene and Capone²¹. Products of DMS metabolism are those reported for methylotrophic and chemolithotrophic metabolism^{9,10}. Diffusion and advection are ignored.

microbial consumption of DMS is tightly coupled with its production.

The impact of grazing on algal and bacterial populations must also be included in considerations of climate-change effects on DMS. Dacey and Wakeham¹⁹ have shown that an important pathway for DMS production is likely to be through organisms that graze DMSP-producing algae, and those that either metabolize DMSP themselves or liberate dissolved DMSP (DMSP_{diss}). Assuming that conversion of DMSP_{diss} to DMS is relatively rapid (R.P.K., manuscript in preparation), gross DMS production will be a function of the grazing pressure on the DMSP-producing algal population, which may or may not be the same as the grazing pressure on the algal population as a whole. This is likely to depend on the size classes of the DMSP-producing algae and on any preference or avoidance of these algae by grazers. These latter points could be critical to our understanding of DMS cycling if the mechanisms of DMS production differ in systems dominated by large zooplankton grazers compared to those dominated by nanoplanktonic grazers.

Similarly, if DMS and DMSP_{diss} metabolism occur in the bacterial populations, these activities will probably be controlled by the concentrations of the respective substrates and by bacterial mortality (most likely through nanoplanktonic grazing and possibly by virus infection²⁰). Thus, DMS concentrations are intimately connected to the structure of the marine pelagic food web, particularly the microbial loop. Plankton populations that interact to affect DMS concentrations (such as zooplankton, algae, heterotrophic nanoplankton and bacteria) are not likely to be in a steady state, and therefore predicting effects of climate change on DMS will involve complex models that consider ecological interactions as well as physical and chemical factors. Our ability to use such models will require far greater understanding of individual processes than we now have. □

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A record from Lake Cardiel of climate change in southern South America

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SOUTHERN South America, the only continental land mass lying between 38° S and the Antarctic Circle, presents a rare opportunity to reconstruct terrestrial records of palaeoclimate in an area that is subject to shifts of polar and mid-latitude wind and pressure belts. In the bulk of this region (Patagonia) are many hydrographically closed lakes which, despite the palaeoclimatic significance of the region and the proven utility of lake level records in reconstructing past climates^{1,2}, have escaped detailed study. Using sedimentary and geomorphic evidence, together with ¹⁴C dating, we present here a detailed lake level record for Lake Cardiel (Fig. 1). The highest level of the past >21,000 years occurred at about 9,780 BP, reminiscent of tropical lakes rather than those in northern mid-latitudes^{1,2}. The Lake Cardiel record corroborates and clarifies the timing of the wetness record inferred from pollen studies in Patagonia.

Lake Cardiel is a large (360 km²) water body that occupies a xeric ~4,000-km² closed depression east of and hydrographically isolated from the Andes in southern Argentina (Fig. 1). Strand lines encircle the lake at elevations as much as 75 m above today's shoreline, attesting to former higher levels³. These appear to be neither warped nor faulted. Littoral embankments at and below the +55-m level are prominent. They are composed of unvarnished clasts and are breached only where they intersect the most active streams and arroyos. By contrast those above +55-m are subdued, deeply dissected, only traceable around <30% of the lake and often are covered with stone pavements composed of varnished beach shingle, indicating that they are at least several times older than the lower ones.

In the course of the fluctuations that deposited the embankments the lake alternately embayed and abandoned the lower reaches of the tributary canyons. During transgressions and highstands the streams built deltas in the canyons, whereas at low lake levels deltas formed beyond the canyon mouths. In response to the modern drop in lake level the streams have incised their deltas, exposing alternating sequences of transgressive and regressive deposits with intervening soils. In some cases these can be traced for thousands of metres up and down stream; in several instances individual sequences terminate at a buried or surface strand line that marks the terminal elevation of a transgression.

Datable materials, including tufa (lacustrine deposits of CaCO₃) and organic detritus, abound in these sections. The tufa deposits occur as beachrock (tufa-cemented littoral sediments), and as rinds that coat the outside of littoral cobbles and boulders. We believe that ¹⁴C assays on these littoral tufa deposits provide reasonable estimates of shoreline age because: (1) modern algae from the lake yield a $\Delta^{14}\text{C}$ of 108‰ modern (LDGO 1735 E, Table 1), indicating that the lake does not act as a significant reservoir for old carbon; (2) at one sub-surface exposure, tufa, dated at 9,780 ± 80 BP (LDGO 1735 O), is immediately overlain by *Chara* that dates at 9,480 ± 95 BP (ETH 5210). This suggests that secondary (post-depositional) contamination of tufa has been minimal, at least during the past 10 kyr.

Tufa also occurs as thin plates intercalated in laminated lacustrine sediments. In most cases ¹⁴C assays on these tufa plates seem to provide reasonable ages of the host sediments. In two instances, however (both involving previously existing sediments that were reinundated), the tufa plates are clearly younger than the host sediments. We therefore assign less authority to dates from tufa plates, and assume that they provide only minimum ages on the host sediments.

Sections measured and logged along the Cardiel River, the Bayo River and Arroyo Cerro Gorro during the austral summers of 1987-88 and 1988-89 have been generalized in Fig. 1. Figure 2 depicts the inferred lake level fluctuations.

The oldest transgressional unit exposed in the walls of the stream cuts is composed of laminated silts and clays (Unit 1). Tufa collected from the underlying cobbles dates at 31,400^{+1,300/-1,100} yr BP (LDGO 1714 X). Because of the antiquity of the tufa, and a lack of corroborating dates, we consider this a minimum age. Unit 1 can be traced to as high as +60 m in Arroyo Cerro Gorro. It may terminate and merge with the littoral embankment at +78 m, but this relationship is not proven. Unit 1 is overlain and partially truncated by alluvial sands and gravels (Unit 2) that are traceable lakeward to the +11-m level, indicating that the lake declined to or below that elevation during the regression following ~31,400 yr BP.

Evidence for a second transgression consists of a localized deposit of lacustrine silt (Unit 3) that overlies Unit 2. Tufa collected from the base of the silts at +12-m dates at 20,700 ± 320 yr BP (LDGO 1714 B), establishing a minimum date for the beginning of the transgression. Neither the timing nor the

FIG. 1 Index map and composite stratigraphic section. Stratigraphy is generalized from sections exposed in stream cuts. Heavy lines represent regressional units and soils; areas between lines are transgressional units. No attempt has been made to portray realistically the unit thickness. Unit designations (circled numbers) are provided for discussion purposes only. Uncircled numbers are dates (yr BP).

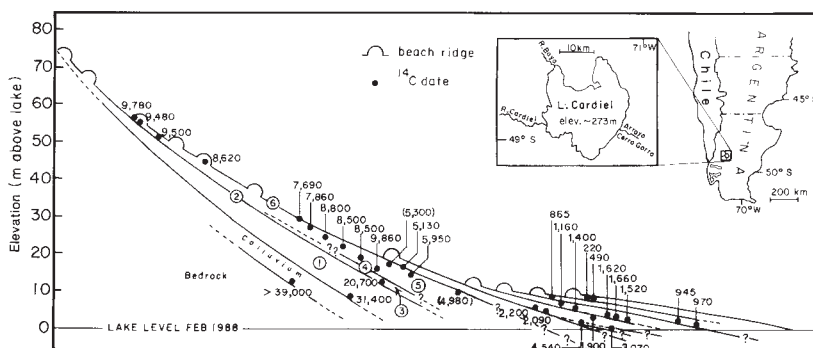


TABLE 1 Radiocarbon dates from Lake Cardiel

Sample No.	Date (yr BP)	Sample description	Sample location	Significance
LDGO 1714 F	>39,000	Charcoal from stump buried in basal colluvium	Right channel wall, Cardiel R, elev. +10 m	Provides minimum age on oldest Quaternary deposit exposed in stream cuts
LDGO 1714 X	31,400 ^{+1,300} _{-1,100}	Lithoid tufa at base of lacustrine silts	Left channel wall, Cardiel R, elev. +8 m	Provides minimum age on commencement of lake transgression
LDGO 1714 B	20,700 ± 320	Lithoid tufa low in lacustrine silts	Left channel wall, Cardiel R, elev. +13 m	Dates commencement of lake transgression
LDGO 1735 D	9,860 ± 110	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. +12 m	Lake approaching highstand of +55 m
LDGO 1735 O	9,780 ± 80	Marly tufa on newly inundated stream gravels	Left channel wall, elev. +52 m, A. Cerro Gorro	Lake reaches elevation of ~+53 m
LDGO 1735 M	9,500 ± 100	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+45 m	Lake near highstand of +55 m
ETH 5210	9,480 ± 95	<i>Chara</i> detritus from basal lagoonal clays	Left channel wall, A. Cerro Gorro, elev. +53 m	Immediately postdates lake stand of +55 m
LDGO 1735 H	8,800 ± 100	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+26 m	Lake remains above +26-m level
LDGO 1735 R	8,620 ± 70	Tufa-cemented beach shingle	+43-m embankment on east shore	Dates recession to the +43-m level
LDGO 1735 F	8,500 ± 70	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+25 m	Lake remains above +25-m level
LDGO 1735 G	8,500 ± 70	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+23 m	Lake remains above +23-m level
LDGO 1735 J	7,860 ± 80	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+7 m	Lake remains above +27-m level
LDGO 1714 A	7,690 ± 80	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+28 m	Receding lake approaches +28-m level
LDGO 1735 P	5,950 ± 60	Charcoal from within A-horizon	Right channel wall, Cardiel R, elev. ~+12 m	Provides maximum age on lake transgression; provides check on date LDGO 1714 C
LDGO 1735 K	5,300 ± 60	Plates of lithoid tufa in lacustrine sandy silts	Left channel wall, Cardiel R, elev. ~+15 m	Tufa intercalated within early Holocene sediments that were reinundated ~5,100 BP
LDGO 1714 E	5,130 ± 50	Woody detritus from surface of O-horizon	Surface of buried soil, left channel wall, Cardiel R, elev. +16 m	Dates mid-stages of lake transgression
LDGO 1714 C	4,980 ± 40	Plates of lithoid tufa in lacustrine sandy silts	Right channel wall, Cardiel R, elev. ~+12 m	Lies stratigraphically below sample of date 5,950 BP, thus questionable. Intercalated in early Holocene sediments that were reinundated ~5,100 BP
LDGO 1714 R	4,540 ± 60	Outside rings of stump rooted in cobble gravel	Right channel wall, Cardiel R, elev. +3.7 m	Provides max lake level for decades before 4,540 BP; dates minor(?) transgression
LDGO 1714 O	3,070 ± 60	Compressed organics in deltaic sandy gravels	Left channel wall, Cardiel R, elev. +2.3 m	Dates minor(?) transgression to +2.3-m level
LDGO 1714 L	2,200 ± 50	Outside rings of rooted stump	Left channel wall, Cardiel R, elev. +6 m	Dates mid stages of lake transgression
LDGO 1714 J	2,090 ± 30	Compressed peat from base of lacustrine silts	Left channel wall, Cardiel R, elev. +4.5 m	Dates early stages of lake transgression
LDGO 1714 P	1,900 ± 60	Charcoal from human occupation site	Buried soil, right channel wall, Cardiel R, elev. +6 m	Lake stands below +6-m level
LDGO 1707 D	1,660 ± 40	Outermost wood from rooted stump	Buried soil, right channel wall, Cardiel R, elev. +4 m	Dates mid stages of lake transgression
LDGO 1714 D	1,620 ± 40	Outermost wood from rooted stump	Buried soil, right channel wall, Cardiel R, elev. +5 m	Dates mid stages of lake transgression
LDGO 1714 W	1,520 ± 40	Outermost wood from rooted stump	Buried soil, left channel wall, Cardiel R, elev. +3 m	Dates early stages of lake transgression
LDGO 1714 U	1,400 ± 40	Outermost wood from rooted stump	Buried soil, right channel wall, Cardiel R, elev. +4 m	Dates mid stages of lake transgression
LDGO 1714 S	1,160 ± 30	Organic detritus from surface of O-horizon	Surface of buried soil, right channel wall, Cardiel R, elev. +7 m	Dates mid stages of lake transgression
LDGO 1714 I	970 ± 30	Organic detritus from surface of O-horizon	Surface of buried soil, right channel wall, Bayo R, elev. +10.5 m	Dates late stages of lake transgression
LDGO 1714 N	945 ± 50	Compressed organic detritus from deltaic sediments	Left channel wall, Cardiel R, elev. +2 m	Dates early stages of lake transgression
LDGO 1714 H	865 ± 40	Outermost wood from rooted stump	Buried soil, right channel wall, Bayo R, elev. +10 m	Dates late stages of lake transgression
LDGO 1714 V	490 ± 30	Compressed organic detritus in lake sediments	Right channel wall, Cardiel R, elev. +6 m	Dates mid stages of lake transgression
LDGO 1714 AA	260 ± 30	Outermost wood from rooted stump	Left channel wall, A. Cerro Gorro, elev. +50 m	Dates transgression-induced(?) stream aggradation that buried soil and stump
LDGO 1707 A	220 ± 60	Compressed organic detritus in lake sediments	Right channel wall, Cardiel R, elev. +6 m	Dates mid stages of lake transgression
LDGO 1714 Y	¹⁴ C/C 108% modern	Modern lake algae	Eastern shore, Lake Cardiel	Approximate radiocarbon of modern lake water

elevation of the resultant highstand has been determined, but it is clear that this transgression never attained the +55-m level or, tentatively, the +30-m level. Following this rise, Lake Cardiel receded to or below +11 m, as inferred from the lowest known elevation of the alluvial gravels of Unit 4.

The lake regression after 20,700 yr BP was followed by a large-magnitude transgression that is recorded in the stream cuts as >2 m of laminated silts and clays (Unit 5). Along Arroyo Cerro Gorro this unit, coarsening to silts and sands at progressively higher elevations, can be traced nearly continuously to

+52 m. At that level it grades into a 3-m-thick embankment of beach shingle. The well developed soil profile that lies above the +55-m level, and the obviously greater age of the littoral embankments that stand above that elevation, provide compelling evidence that the +55-m embankment marks the highstand of this transgression.

The embankment at +55 m dammed the Cerro Gorro Canyon, creating a shallow-water lagoon in which accumulated laminated clays replete with *Chara* remains. Immediately upstream from the base of the embankment a ~6-m-thick exposure in the channel wall reveals the following sequence of sediments: riverine gravels grade upwards into deltaic foreset gravels, which in turn fine upwards into littoral sands, then into the *Chara*-rich clays of the lagoon. Marly tufa deposited as a single horizon on the upper surface of the delta gravels dates at $9,780 \pm 80$ yr BP (LDGO 1735 O). This date applies to the final stages of the transgression. *Chara* detritus extracted from low in the overlying lagoonal clays yields an accelerator-mass-spectrometry date of $9,480 \pm 95$ yr BP. This assay immediately postdates the +55-m highstand.

At this level the surface area of the lake was ~30% greater than today. At equilibrium, this highstand would have required an effective inflow ~30% greater than that of the present day. (This is an underestimate of the difference between past and present inflow, because the lake today continues to shrink dramatically in response to modern hydroclimatic conditions.)

As the lake receded from the +55-m highstand it deposited embankments at the +49-m, +43-m, and +34-m levels. Along the eastern shorelands, portions of the +43-m embankment are thoroughly cemented with tufa. This beachrock dates at $8,620 \pm 70$ BP (LDGO 1735 R), thus establishing the timing of the first 12 m of regression from the +55-m highstand.

In the walls of the stream cuts, the post-9,780-BP lake regression is expressed as up to 7 m of laminated to thin-bedded fine sands and silts (Unit 6) that overlie the silts and clays of the transgression. This regressional sequence is capped by a discontinuous veneer of beach gravels. Tufa plates collected from five different horizons in the regressional sediments range in age from $9,860 \pm 110$ yr BP (LDGO 1735 D) (near the base of Unit 6) to $7,690 \pm 80$ yr BP (LDGO 1714 A) (near its top at an elevation of +28 m). Only one minor reversal characterizes this progression of dates. Although only the uppermost of the dates can arguably be tied to an actual lake level, the suite of assays, in concert with the known age of the +43-m embankment, suggests that the lake remained above the +28-m level

until shortly after ~7,700 years ago.

None of the sedimentary sections exposed in the stream cuts appear to contain deltaic deposits dating from between ~7,700 and ~5,100 yr BP. Given the nature and distribution of the existing sedimentary exposures, it seems likely that, had the lake risen to elevations above the +10-m level during this interval, it would have left a lasting record of the transgression. We tentatively conclude, therefore, that the lake remained below +10 m, and that it may have fallen to levels below the present-day lake surface, between ~7,700 and ~5,100 yr BP.

Over the past 5,100 years Lake Cardiel has undergone at least five transgressions and regressions. In all cases the transgressions are expressed as lacustrine sediments overlying soils that developed on older regressional deposits. Sixteen ^{14}C dates, on woody detritus and charcoal collected from the buried soils, and on wood from stumps that remain rooted in the soils, constrain the dates of these fluctuations. Although the first of the rises (culminating ~5,130 $\pm$ 50 yr BP, LDGO 1714 E) was substantial (to +21.5 m), at no time during the past five millennia has the lake surface approached the highstand of ~9,500–9,800 BP. Several of the regressional units of the past 5,100 years closely approach (and in two cases extend to below) the present-day lake surface. Based on the apparent absence of the artefacts on and below the embankment associated with the most recent highstand (at +8.9 m), we conclude that the lake began its final regression after the disarticulation of indigenous culture, placed by archaeologist Carlos Gradin (personal communication) at AD 1910–20.

The early Holocene highstand of Lake Cardiel points to hydroclimatic conditions effectively wetter than at any other time during the past >21,000 years—a conclusion in keeping with palynological studies in Patagonia, Tierra del Fuego and Chile^{4–6}. Markgraf's^{3,6} palynological compilation from southern Patagonia also corroborates the middle and late-Holocene portions of the lake curve. She places a wet interval at ~5,000 yr BP (coinciding with a lake rise of >20 m) which was both preceded and followed by 2,000–3,000 years of relatively dry climate (coinciding with lowstands of Cardiel). According to Markgraf, the mixed-vegetation types of the past ~2,500 years point to rapid (but undefined) swings of climate (reflected in the Lake Cardiel record as four century-scale episodes of moderate wetness, alternating with four dry episodes). The sensitivity and datability of the Lake Cardiel record thus makes it possible to clarify and enhance the pollen-based palaeoclimate record from southern South America.

The early Holocene behaviour of Lake Cardiel resembles more closely lakes of low latitudes than lakes of the Northern Hemisphere mid-latitudes¹. This underscores the complexity of the glacial/post-glacial climate transition which, although generally displaying global synchrony in temperature trend⁷, was characterized by marked regional differences in the timing and sign of wetness changes. The south Andean glaciers seem to have been smaller than at present throughout the early Holocene⁸, suggesting that conditions in southern Patagonia may have been warm as well as wet. Although the cause of early Holocene warmth and wetness in Patagonia must be left to modellers, we suggest that the abnormally high temperature of sub-Antarctic surface waters ~9,000–10,000 BP^{9,10}, coupled with a greater incidence of easterly winds than exists today, may help explain the phenomenon. □

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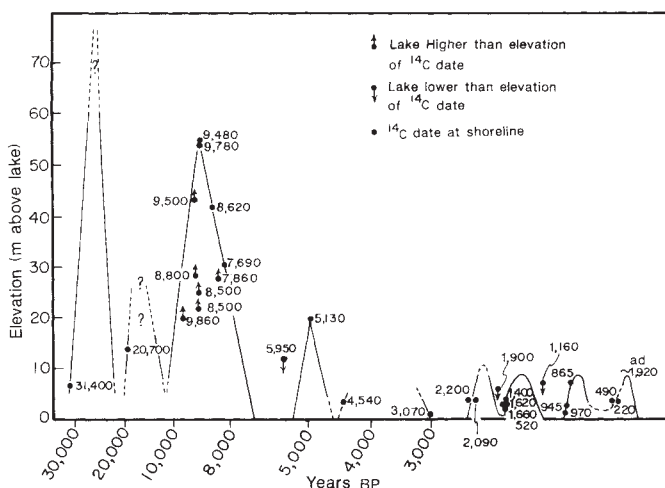


FIG. 2 Late Quaternary fluctuation curve for Lake Cardiel. Numbers are dates (yr BP, except where stated AD).

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Eustatic sea level fluctuations induced by polar wander

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THE observation of apparently simultaneous episodes of deposition or non-deposition of marine sediments in different parts of the world has led to the proposal that at least some sea level rises and falls must be global, or eustatic, in character^{1–3}. Here we show that polar wander of a viscoelastic stratified Earth can induce global sea level fluctuations comparable to the short-term component in the eustatic sea-level curves. The sign of these fluctuations, which are very sensitive to the rheological stratification, depends on the geographical location of the observation point; rises and falls in sea level can thus be coeval in different parts of the world. This finding is in distinct contrast to the main assumption underlying the reconstruction of eustatic curves, namely that global sea-level events produce the same depositional sequence everywhere. This apparent contradiction is due to the poor time resolution of the stratigraphic records in the distant past, which is comparable to the timescale of polar motion^{4–6}, and to non-uniform data coverage. We propose that polar wander should be added to the list of geophysical mechanisms (the others are glacial instabilities, plate tectonic mechanisms, sea-floor spreading, and thermal and compaction-induced subsidence) that can control the third-order cycles in sea level.

Sea level fluctuations, due to variations in the centrifugal force associated with long-term wander of the Earth's rotation axis, can be predicted theoretically from a radially stratified viscoelastic Earth model⁷. The possible causes for these long-term displacements of the axis of rotation can originate in the mantle, as suggested by the rotational responses of the Earth to the anomalous density heterogeneities inferred from seismic tomography^{8,9}, or can be caused by the surface redistribution of melt water from ice ages¹⁰. Here we shall disregard the possible dynamical sources of polar wander and focus instead on the effects induced by rates of polar motion, the amplitude of which is selected to be in agreement with recent analyses of palaeomagnetic data¹¹. In the Laplace transform domain, the perturbation in the centrifugal potential depends, to first order, on the direction cosines of the rotation axis $m(s) = m_1(s) + im_2(s)$

$$\psi(s) = -\sqrt{2\pi/15}\Omega^2 a^2 m(s) Y_2^1(\theta, \phi) \quad (1)$$

where Ω is the rotation rate of the Earth, a is the Earth's radius, Y_2^1 is the spherical harmonic and θ, ϕ are the colatitude and longitude, respectively.

Because our model is radially symmetric, and the distribution of the ocean is assumed uniform, this $l=2, m=1$ perturbative potential induces a signal in the relative sea level fluctuation ξ of the same angular degree and order. Lateral variations in viscosity would contaminate this signal with shorter wavelengths. We have then

$$\xi_2^1(s) = (1 + k_2^T(s) - h_2^T(s))\psi(s)/g \quad (2)$$

where k_2^T, h_2^T are the $l=2$ components of the tidal Love numbers for the gravitational potential and vertical displacement respectively and g is the gravitational acceleration. This expression of the sea level change relative to the sea bottom does not account for the effects of self-attraction of the uniform ocean¹². This effect can be easily seen to produce an increase of at most 10 per cent on our results. The term $(1 + k_2^T(s))$ yields the deformation of the ocean surface relative to the Earth's centre, whereas $h_2^T(s)$ controls the vertical displacement of the sea bottom. Owing to the lag between these two contributions, perturbations in the centrifugal potential induce sea level fluctuations, as shown by the drawing in Fig. 1. The white arrow indicates the direction of the polar wander, and the solid and dashed curves depict the shift of the equipotential surface (geoid). Highstands and lowstands are generated, depending on the latitude and longitude of the observation points with respect to the polar motion. To quantify these sea level fluctuations, we consider the spectral decomposition of the tidal Love numbers

$$k_2^T(s) = k_e^T + \sum_{i=1}^M k_i^T/(s - s_i) \quad (3)$$

$$h_2^T(s) = h_e^T + \sum_{i=1}^M h_i^T/(s - s_i) \quad (4)$$

where k_e^T and h_e^T represent the elastic contributions, M denotes the number of relaxation modes associated with the Earth model, s_i and k_i^T, h_i^T their inverse relaxation times and strengths⁷. Our model consists of an uniform elastic lithosphere of 100 km, two viscoelastic Maxwell layers, with viscosities ν_1 and ν_2 , modelling the upper and lower mantle and an inviscid core⁷. The interface separating the upper and lower mantle is located at a depth of 670 km, and appropriate boundary conditions for the normal stress at this interface are used to reproduce the mechanical behaviour of phase-change or chemical transitions, which are assumed fully adiabatic and non-adiabatic respectively^{8,9}. In what follows, we study the responses of the Earth subject to a constant polar wander of 1° Myr^{-1} ; this value is a lower bound, a deduced from palaeomagnetic observations^{6,11} and these results can be extended to higher polar drift rates by linear scaling.

In Fig. 2 the perturbative potential is applied at time $t=0$; we follow the evolution of the sea level at mid-latitudes, where the effects are larger owing to the Y_2^1 dependence of the perturba-

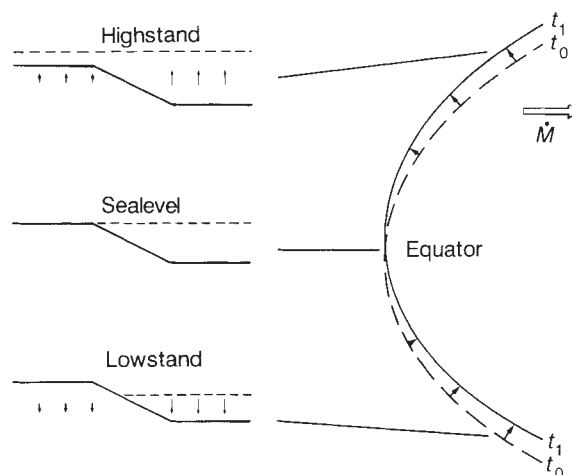


FIG. 1 Pictorial representation of sea level fluctuation induced by polar wander. The white arrow denotes the direction of polar drift, at a rate $M=1^\circ \text{ Myr}^{-1}$. The solid and dashed curves represent the deformation of geoid and topography of the sea floor at times $t=t_0$, when the perturbation is initially imposed and a subsequent time $t=t_1$. Sea level is unperturbed at the Equator but rises and falls occur in the Northern and Southern Hemispheres, respectively.

tion. It is quite remarkable that sea level fluctuations are extremely sensitive to viscosity stratification and lithospheric thickness. These curves are characterized by transient behaviour, more pronounced for high lower-mantle viscosities, followed by a linear trend, which is connected with a constant rate of polar wander¹⁰. For smooth viscosity contrasts, phase-change models (dashed curves), produce a smaller signal than chemically stratified ones (solid curves). For the chemically stratified models, deflections of the 670-km discontinuity induce a buoyant restoring force which inhibits viscous relaxation in the mantle. This, in turn, reduces the vertical uplift of the sea floor and helps to maintain the offset between the sea-floor topography and the geoid. Recent work by Gurnis¹³ has emphasized the importance in the trade-off between the dynamically induced topography and the geoid in the flooding of continental platforms. For high viscosity contrasts ($\nu_2 = 10^{23}$ Pa s), the stiffening of the lower mantle overcomes the dynamical effects associated with the nature of the 670-km discontinuity. Thus phase-change and chemically stratified models exhibit the same behaviour. The dotted curve, corresponding to a model with a reduced lithospheric thickness of 50 km, shows the sensitivity of sea level fluctuations induced by polar wander to variations in lithospheric thickness. Recent analyses of geoid anomalies¹⁴ and post-glacial rebound^{15,16} favour high viscosity contrasts. These models then predict sea level fluctuations of the order of several tens of metres on timescales of 10^6 yr, which are comparable to the third-order cycle in the eustatic curves.

It is useful to analyse the behaviour of the rate of sea level fluctuations (Fig. 3). Rates of the order of 0.05 – 0.1 mm yr⁻¹ are maintained for timescales of 0.5 Myr if the viscosity contrast is sufficiently high ($\nu_2/\nu_1 = 50$ to 100). For uniform models, or mild viscosity contrasts ($\nu_2/\nu_1 = 1$ to 10), rates of sea level fluctuations decay in a few tens of thousand years. After the decay of the initial transient, the rates reach a final value of ~ 0.02 mm yr⁻¹, irrespective of the rheological stratification. As expected, phase-change models produce lower rates, except for

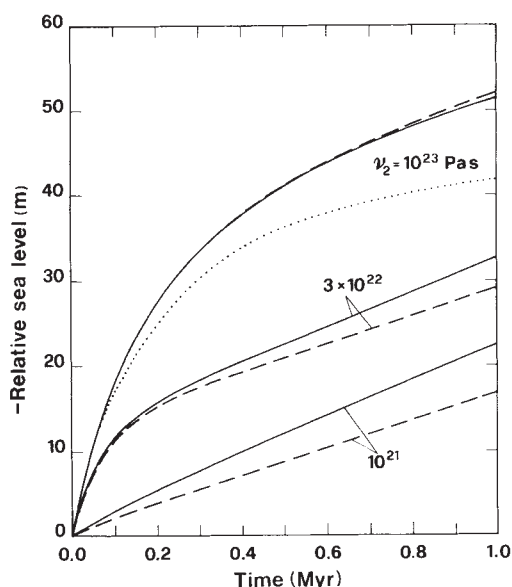


FIG. 2 Time dependence of relative sea level fluctuations at mid-latitudes ($\theta = 45^\circ$ N, $\phi = 0^\circ$), corresponding to $\dot{M} = 1^\circ$ Myr⁻¹ and polar motion toward Greenwich. The perturbative potential is applied at time $t = 0$. Solid curves correspond to chemically stratified models (fully non-adiabatic) and dashed ones correspond to fully adiabatic phase changes. Lithosphere thickness is 100 km, except for the dotted curve, which corresponds to 50 km. The upper-mantle viscosity ν_1 is 10^{21} Pa s, and the lower mantle is varied from 10^{21} Pa s (bottom) to 10^{23} Pa s (top).

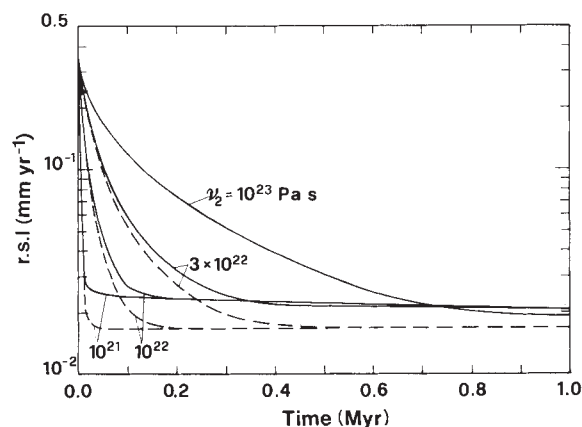


FIG. 3 Time dependence of relative sea level (r.s.l.) in mm yr⁻¹. Solid curves correspond to chemically stratified models and dashed ones correspond to fully adiabatic phase changes. For $\nu_2 = 10^{23}$ Pa s, the chemical transition and phase-change models are indistinguishable. Results for $\nu_2 = 10^{22}$ Pa s are also shown.

high viscosity contrasts, in which case these models are indistinguishable from chemically stratified ones. We find that rates of sea level fluctuations are around one order of magnitude smaller than those associated with vertical deformation of the sea floor which are ~ 1 – 1.5 mm yr⁻¹. This value agrees with the estimates made by Hanada¹⁷ from the readjustment of the Earth rotational bulge, driven by polar wander. Rates of the order of 0.1 – 0.5 mm yr⁻¹ in the initial state of polar wander, are also consistent with that obtained by Han and Wahr¹⁸ who were the first to account for the effects induced by a variable centrifugal potential on post-glacial rebound within the framework of a viscoelastic mantle.

The rates predicted by our model are of the same order of magnitude as the short-wavelength ones associated with temporal variations of the horizontal tectonic stress field in the lithosphere¹⁹. From these results, it is clear that the proposed mechanism of polar wander is efficient in inducing sea level fluctuations especially during epochs of rotational instabilities, caused by mantle flows. Significant shift of the rotation pole occurred, for example, during the Late Cretaceous^{6,11,20}. Other effects from lateral variations in the mantle viscosity structure would act to produce eustatic sea level changes with higher-degree harmonics than $l = 2$. Therefore our findings indicate that the highstand or lowstand system tracts observed in one part of the world are not necessarily correlated with the same depositional sequence in another geographical location. A highstand in a marine package in the Northern Hemisphere can, in fact, be coeval with a lowstand in the Southern Hemisphere at the same longitude if the only active mechanism that induced the eustatic sea-level event was polar wander. There is thus an urgent need to improve the temporal resolution of sequence stratigraphy by about one order of magnitude so that eustatic sea-level events in sedimentary packages from different parts of the world are recognized accurately. □

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Model of carbon fixation in microbial mats from 3,500 Myr ago to the present

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BIOLOGICAL carbon fixation is an important part of global carbon cycling and ecology. Fixation that took place 3,500 million years ago is recorded in the laminated sedimentary rock structures known as stromatolites, which are fossilized remains of microbial mat communities^{1–4}. Stromatolites are the most abundant type of fossil found in the Proterozoic (2,500 to 590 Myr ago), but they then declined, possibly because of predation and competition^{5,6}. Using modern microbial mats as analogues for ancient stromatolites, we show that the rate of carbon fixation is higher at the greater levels of atmospheric CO₂ that were probably present in the past⁷. We suggest that carbon fixation in microbial mats was not carbon-limited during the early Precambrian, but became carbon-limited as the supply of inorganic carbon decreased⁸. Carbon limitation led to a lower rate of carbon fixation, especially towards the end of the Precambrian. Thus, another reason for the decline of the stromatolites could have been a decrease in available CO₂.

The microbial mat systems studied are located in evaporation ponds of the salt company Exportadora de Sal, SA (Fig. 1). One study site was located in evaporation pond 5 (2,295 hectare, ~1 m in depth, 5–10% NaCl) ~2 km east of the outflow gate from pond 5 to 6. The bottom of pond 5 is covered by a laminated microbial mat consisting of, from top to bottom, diatoms (0–2 mm) (L.J.R., unpublished observations), cyanobacteria dominated by *Microcoleus chthonoplastes* (1–2 mm), and layers composed of prokaryotes living heterotrophically under anaerobic conditions (~3 cm) (refs 9–11). The other mat communities live in the intertidal area of Laguna Ojo de Liebre (Fig. 1). Both were cyanobacterial, one dominated by *Lyngbya aestuarii*, the other by *Calothrix crustacea* (~2–5 mm in thickness).

Initial experiments were conducted on 2–4 June 1989 in all three mat communities, and repeated for the *Lyngbya* mats on 2 November 1989. Carbon fixation rates were obtained under different levels of dissolved inorganic carbon (DIC) ranging from ~2 mM (ambient) to 252 mM. Environmental parameters during the experiments are described in Table 1. We conducted all experiments during the middle of the day when light levels were essentially constant and, to insure that our observations were not the result of a light-level effect, experiments were run at random. Mat samples were incubated *in situ* in bottomless clear glass serum vials (125 ml; cross-sectional area, 17.2 cm²) sealed with a syringe septum. The vials were inserted into the mat in such a way that the bottle was filled with air. Pond or lagoon water (DIC ≈ 2 mM, ~pH 7.2), or water with supplemen-

tary NaHCO₃ (~pH 7.2), was spiked with NaH¹⁴CO₃ (ICN Biomedicals or New England Nuclear) to a final concentration of 1–2 µCi ml⁻¹ and injected into the bottles. Samples were removed periodically at times of up to 60 min and immediately frozen on dry ice. On return to the laboratory, the samples were defrosted, homogenized in a buffer (100 mM Tris buffer, pH 8; 50 mM EDTA) and sonicated on ice. Two aliquots were placed in separate scintillation vials where they were acidified, dried, dissolved in warm water and counted in Ecolume (ICN Biomedicals). All counts were adjusted with internal standards. All experiments were performed in triplicate.

Carbon fixation rates were essentially linear over 60 min, but we did observe variations attributable to such factors as fluctuations in light level due to small clouds passing overhead. Also, experiments on pond 5 mat have shown that within 20 min of fixation, some portion of the newly fixed carbon is remineralized by respiration (data not shown). After this time, the counteracting forces of carbon fixation and respiration make it difficult to determine carbon fixation rates alone. To minimize the impact of respiration on the pool of acid-stable carbon, the shortest incubation time experimentally feasible, 15 min, was used to calculate carbon uptake rates.

Carbon fixation rates were calculated from total acid-stable counts in the duplicate aliquots from each of the triplicate experimental samples, excluding both the highest and lowest count. Carbon fixation rates of pond 5 and *Lyngbya* mats, expressed as µg carbon fixed m⁻² min⁻¹ and graphed as a function of DIC, are shown in Fig. 2a. Results for *Calothrix* mats were similar (data not shown). Standard deviations for each rate measurement averaged 11% for *Lyngbya* samples and 15% for pond 5. Carbon fixation rates for pond 5 and *Lyngbya* mats attain a maximum at ~50 mM DIC. These data are consistent with studies¹² on a pure culture of the cyanobacterium *Synechococcus* which showed that photosynthesis probably becomes saturated with respect to inorganic carbon at DIC levels just over 25 mM.

Current astrophysical models suggest that the Sun has increased in luminosity since the origin of the Solar System. To account for the presence of liquid water on the early Earth in spite of a lower solar luminosity, Owen *et al.*⁷ proposed that a higher level of CO₂ in the past created a greenhouse effect, thus raising the surface temperature of the Earth. If this widely accepted hypothesis is correct, it implies correspondingly higher levels of DIC in ancient oceans. Pollack *et al.*¹³ have calculated the DIC levels in a hypothetical ocean as a function of pCO₂, assuming an isothermal ocean (5 °C) with a salinity of 3.5%

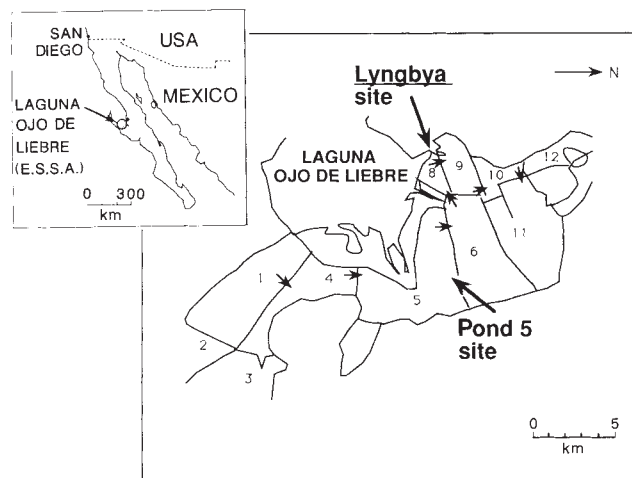


FIG. 1 Map showing location of study sites. Small arrows indicate the direction of water flow through the gates connecting evaporation ponds.

TABLE 1 Environmental conditions for carbon fixation experiments

Mat type	Date	Time (h)	Light ($\mu\text{E s}^{-1} \text{m}^{-2}$)	Temperature at mat surface ($^{\circ}\text{C}$)
Pond 5	1 June 1989	11:08–13:35	1820–1876	23
<i>Lyngbya</i> (in water)	4 June 1989	11:50–15:05	1568–1854	23
<i>Lyngbya</i> (in water)	2 November 1989	11:51–13:02	1176–1400	34
<i>Lyngbya</i> (in air)	1 December 1989	11:20–12:05	1060–1116	24–34

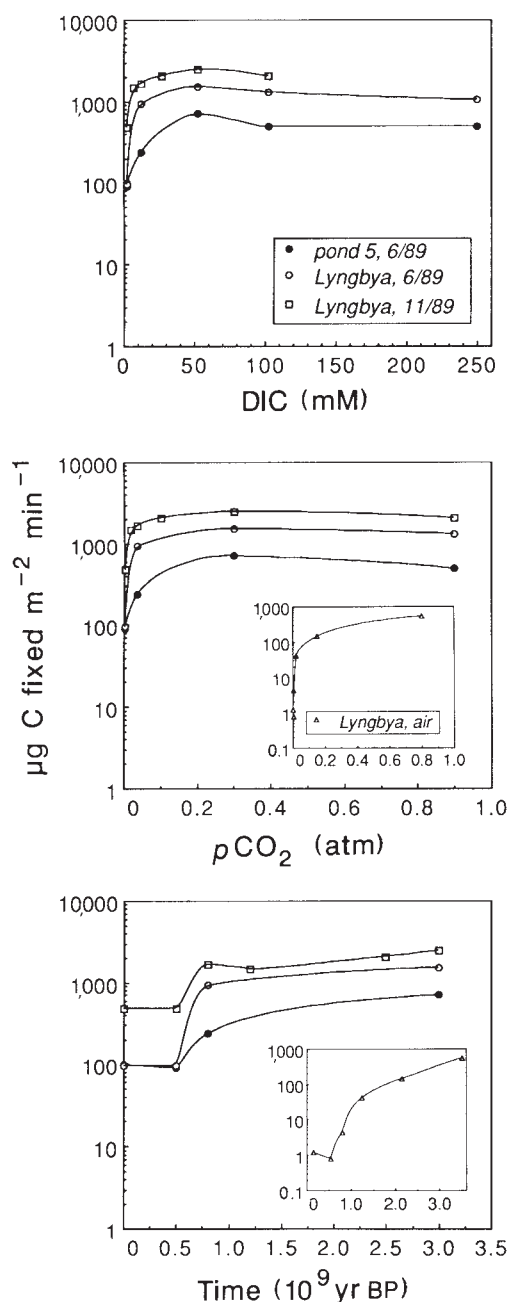


FIG. 2 Carbon fixation rates as a function of dissolved inorganic carbon (DIC), p_{CO_2} , and geological time. *a*, *In situ* determinations of carbon fixation rates as a function of DIC for *Lyngbya* and pond 5 microbial mat communities. Standard deviations for each rate measurement averaged 11% for *Lyngbya* samples and 15% for pond 5. *b*, Carbon fixation rates from *a* expressed as a function of p_{CO_2} . Insert shows carbon-fixation data obtained on moist *Lyngbya* mat. *c*, Carbon fixation rates expressed as a function of geological time for submerged pond 5 and *Lyngbya* mat, and for moist *Lyngbya* mat (insert). Error in these values owing to uncertainty in the model of Kasting⁸ would, at worst, suggest no decrease in fixation rates until ~1,000 Myr BP or a straight logarithmic decline until the Cambrian.

(equivalent to today's oceans), saturated with respect to calcite (CaCO_3), with a concentration of dissolved calcium ions of 10.3 mM (as in the present ocean) and 0.5 km in depth. Under such conditions, they derive a DIC level of 1.27 mM from a p_{CO_2} of 3.4×10^{-4} atm, approximately the atmospheric level of p_{CO_2} found on Earth today and the DIC level found in Laguna Ojo de Liebre and pond 5 (1.6–2.5 mM, ref. 10). For a p_{CO_2} of 5 atm, a level of the same order of magnitude as proposed by Walker¹⁴ and Kasting⁸ for the early Earth, Pollack *et al.*¹³ derive a DIC level of 408 mM.

The model of Pollack *et al.*¹³ allows us to express our experimentally derived carbon fixation rates (Fig. 2*a*) as a function of p_{CO_2} (Fig. 2*b*). To identify p_{CO_2} levels at various points in geological time, we have used the 'best guess' model of Kasting⁸ for the evolution of atmospheric p_{CO_2} . The levels of p_{CO_2} during the Earth's history that are consistent with known patterns of glaciation and liquid water range from ~ 1 bar at 4,500 Myr BP (before present) to 0.03–0.3 bar at $\sim 2,500$ Myr, to 10^{-3} – 10^{-2} bar at 800 Myr. Therefore, we have converted our microbial-mat carbon fixation rates from μg carbon fixed $\text{m}^{-2} \text{min}^{-1}$ as a function of DIC, to carbon fixation rate as a function of p_{CO_2} , and finally to carbon fixation rate as a function of geological time (Fig. 2*c*).

There are two sources of uncertainty in the model presented in Fig. 2*c*. First, our calculations rely on two published models, each contributing uncertainty. For example, there are uncertainties in gas partitioning between the ocean and the atmosphere in the model of Pollack *et al.*¹³ and in the p_{CO_2} values derived by Kasting⁸. Second, our results are based on submerged microbial mats, although many ancient^{3,4} and modern (those found in Shark Bay, Western Australia, for example) stromatolites are intertidal. To address the latter uncertainty, the effect of p_{CO_2} on carbon fixation was determined on *Lyngbya* while the mats were moist (but not submerged) by adding gaseous inorganic carbon. Dried mat samples were soaked in 3.5% NaCl overnight. The excess saline was drained, and triplicate cores (5 cm^2 in area) were placed in clear glass jars. Solutions of NaHCO_3 + $\text{NaH}^{14}\text{CO}_3$ were acidified, mixed and added quickly to the incubation jars before sealing. Samples were incubated simultaneously for 45 min (Table 1), then processed as described above. The carbon fixation rates determined were converted from p_{CO_2} (insert, Fig. 2*b*) to geological time (insert, Fig. 2*c*) using the model of Kasting⁸. The results are similar to those obtained with submerged mats.

Several features of Fig. 2*c* are striking. Maximal carbon fixation rates for the *Lyngbya* and pond 5 mats correspond to $\sim 3,500$ Myr. As stromatolites first appear in the fossil record at 3,500 Myr (ref. 4 and references therein), our data suggest that carbon fixation rates were a maximum shortly after they arose. Carbon fixation rates then began to decrease and plummeted during the final few million years of the Precambrian. Eukaryotes are thought to have arisen by 1,500 Myr¹⁵, and the metazoa by 680 Myr¹⁶, times which correspond to the initial decrease and sharp final decrease in carbon fixation rates presented here. The sudden drop in abundance in the fossil record of stromatolites at the end of the Precambrian coincides with the origin and evolution of metazoan grazers, a correlation that has suggested to previous workers^{5,6} that the decline in stromatolites was caused by metazoan grazing. Although we do not question

that this event had an impact on the abundance of stromatolites, our data suggest that a decrease in carbon fixation rates as a result of the decreased availability of atmospheric inorganic carbon, and thus DIC, also had a role in the sharp decline in stromatolites at the end of the Precambrian.

Although our model emphasizes the relationship between decreasing levels of atmospheric p_{CO_2} over geological time and decreasing rates of carbon fixation, atmospheric O_2 has increased since 3,500 Myr (see, for example, ref. 8). Because O_2 is a competitive inhibitor of CO_2 for the carboxylation reaction catalysed by the photosynthetic carbon fixation enzyme, ribulose 1,5-bisphosphate carboxylase, any increase in p_{O_2} should decrease carbon fixation rates. Thus, all other things being equal, rising levels of oxygen should enhance the effects presented. Other possible environmental factors that are known to affect carbon fixation rates are nitrogen and phosphorus levels. The impact of these factors was determined on both the pond 5 and *Lyngbya* mats by conducting carbon-fixation experiments as described above with the addition of supplemental phosphate, ammonia or nitrate. Carbon fixation in these mats is not, for the most part, limited by these nutrients. When 100 mM nitrate was added in pond 5 (31 October to 1 November 1989), however, there was a maximum of 25% enhancement of carbon

fixation during the middle of the day. This result does not alter the conclusion that, for these mats, the availability of inorganic carbon has a profound impact on the rate of carbon fixation. □

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New hominid skull material from the late Miocene of Macedonia in Northern Greece

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MIocene hominoid material is very scarce^{1–7} and has previously only been reported as cranial fragments in the Old World. Here we describe a new specimen of *Ouranopithecus macedoniensis*, which consists of the right part of the face of an adult male with a portion of the frontal bone, a portion of the left part of the face and the maxilla with complete dentition except the right third molar. The characters of this specimen are not congruent with those of *Sivapithecus* and the pongids, but are more primitive and plesiomorphic for the recent hominid clade (*Gorilla*, *Pan* and *Homo*). The dental characters differ morphologically and metrically from those of the recent great apes and fit better with *Australopithecus afarensis*⁸. *Ouranopithecus* now seems the best candidate forerunner of the Plio-pleistocene Homininae (*Australopithecus* and *Homo*). This specimen was discovered in September 1989, in the late Miocene deposits of central Macedonia (G.K., L. de B. and G.B.), and prepared by G.K. in Thessaloniki and G. Mouchelin in Poitiers. It comes from the new locality of Xirochori in the red sandstone of the Nea Messimbria formation⁹. The fossil is the property of the University of Thessaloniki, Greece (catalogue number XIR-1).

Mandibles and maxillae of bovids, giraffe and mastodonts were associated with the face of XIR-1 (see Fig. 1). This fauna is similar to other faunas from Macedonia found in two other localities of the same geological formation—Ravin de la Pluie (RPI) and Ravin des Zouaves no. 1 (RZ1). These faunas are assigned to the Vallesian Land Mammal Age on the basis of faunal correlation with other sites. It is impossible to date the

TABLE 1 Character measurements of XIR-1

Dental measurements (mm)			
Tooth no.	Definitions	Left	Right
I ¹	MD	10.0	10.8
	La-Li	9.3	9.3
I ²	MD	6.4	6.5
	La-Li	8.0	7.8
C	max.MD	13.3	13.5
	Tr	13.2	14.3
P ³	MD	8.1	8.2
	Bu-Li	13.0	12.4
P ⁴	MD	9.0	8.1
	Bu-Li	13.5	13.2
M ¹	MD	13.0	13.2
	Bu-Li	14.3	14.4
M ²	MD	14.8	15.0
	Bu-Li	15.5	14.9
M ³	MD	14.9	?
	Bu-Li	15.9	?
Facial measurements (mm)			
Glabella to nasion		9.9	
Alveolar to nasion		95.0	
Alveolar to naso-spinal		?	
Nasal aperture height		?	
Nasal aperture maximum breadth		35.0	
Orbite height		30.0	
Orbite breadth		33.0	
Orbito-alveolar height		44.1	
Minimum bi-orbital breadth		26.0	
Bi-orbital breadth across lacrymal crests		24.0	
External bi-orbital breadth		~129.0	
Palatal length		96.0	
Palatal breadth at M ²		~42.0	

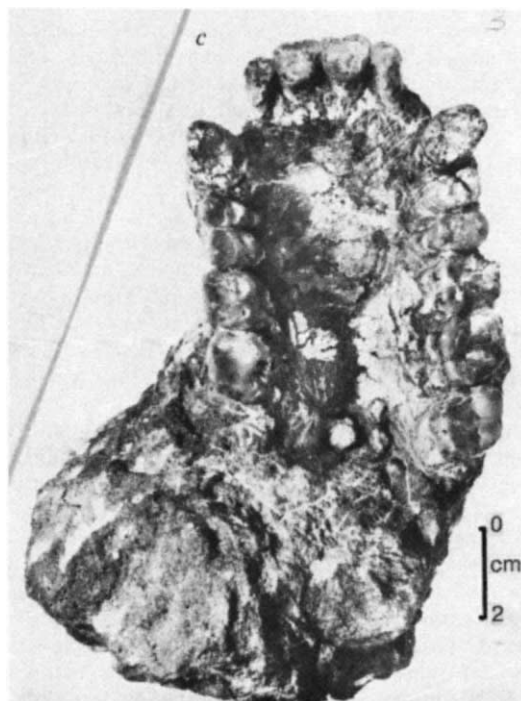
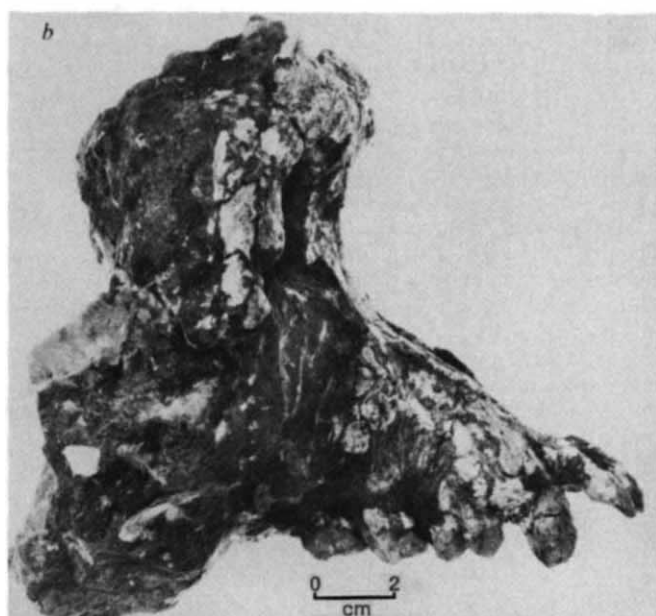
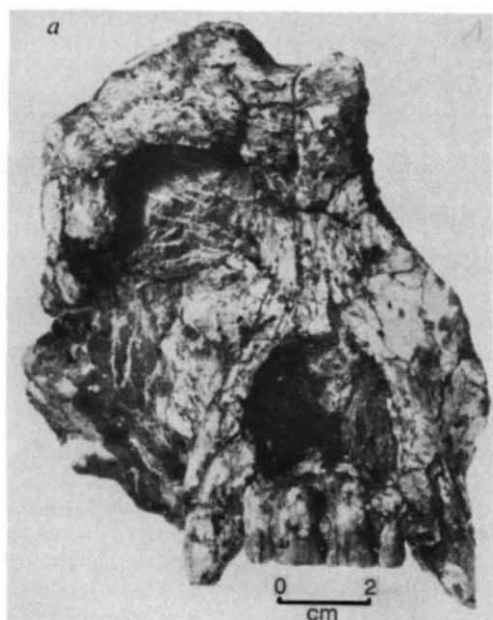
Bu-Li: buccal-lingual diameter; La-Li: labial-lingual diameter; MD: mesial-distal diameter; max., maximum.

Macedonian deposits directly by radiometric methods but some other localities which have yielded similar faunas have been dated to 9–10 Myr ago (the lower part of the late Miocene). These faunas contain many grazing mammals and can be considered as open environment (savanna) faunas.

The measurements of the dentition are given in Table 1. The right M³ is missing but the other teeth are well preserved, except

for the left M^1 which was partially broken and badly worn during life. The central incisors are larger than the lateral ones. There is a diastema between I^2 and the canine (C) and a very small space between the canine and P^3 . The canine is relatively large, indicating that it belonged to a male specimen. The C/ M^1 length ratio is 94%, in the range of female chimpanzees or gorillas. But there is a large sexual variation in *Ouranopithecus*¹⁰. The canine of XIR-1 is very similar to male specimens of Ravin de la Pluie. The size of the jugal teeth is also similar to male *Ouranopithecus* and we believe XIR-1 is a male (Fig. 2). The size of the canines is smaller than in any recent or Miocene great ape¹⁰. Compared with a male *Proconsul nyanzae*, these canines are half the size relative to the length of the jugal tooth rows.

The jugal teeth, premolars and molars are heavily enamelled, as are *Sivapithecus* or australopithecine teeth. P^3 and P^4 are small relative to the molars and only slightly dimorphic. There is a well characterized wear gradient on the molars; the dentine is largely exposed on M^1 , a little on M^2 and not at all on M^3 .

TABLE 2 Character differences between *Proconsul* and *Ouranopithecus*

<i>Proconsul</i>	<i>Ouranopithecus</i>
Brow ridge absent	Brow ridge present
Large canines	Smaller canines
Honing facet present	Honing face absent
P_3 laterally compressed	P_3 lingually expanded
Upper premolar heteromorphic	Upper premolars more homeomorphic
Long premolars with regard to the molars	Shortened premolars with regard to the molars
No (or few) accessory cusps on M^3	Accessory cusps on M^3

They are moderately high crowned and the cusps are low. The general occlusal pattern is *Australopithecus*-like, but less cuspidated. *Proconsul* differs from XIR-1 by the larger canines, the thin enamel, the large cingulum of the molars and the higher external cusps of the premolars^{1,2} (Table 2). *Sivapithecus* differs from XIR-1 by the larger canines but there are some similarities in the jugal teeth⁷. *Lufengpithecus* has long and narrow incisors and canines¹¹.

The face of the new fossil is well preserved and reasonably complete, but a little distorted by fossilization pressure on the right canine fossa area. It is female gorilla-sized but the prognathism is weaker. There is a brow ridge under the orbits and a glabella prominence. The brow ridge is large but not projecting like those of the gorilla or chimpanzee and it looks more like the brow ridges of some australopithecines. In this area, only the external table of the bone is preserved and it is difficult to know if there is a developed frontal sinus. The orbit is low and rectangular; the long axis is directed outwards and downwards. There is a large inter-orbital region. Large orbital foramina are visible on the edge of the orbits, but the bone is damaged on the two sides in the area of the infra-orbital foramina. The nasal bones are flattened and enlarged at their distal extremities. The nasal aperture is broad and the sides converge slightly towards the nasal bones. The canine fossa is present on the right maxilla. The naso-maxilla region is incomplete. Most of the upper part of the premaxilla has been destroyed, but on the left part the level of the premaxillary is much higher than the nasal floor,

FIG. 1 a, Facial view of XIR-1. b, Lateral view of XIR-1. c, Palatal view of XIR-1.

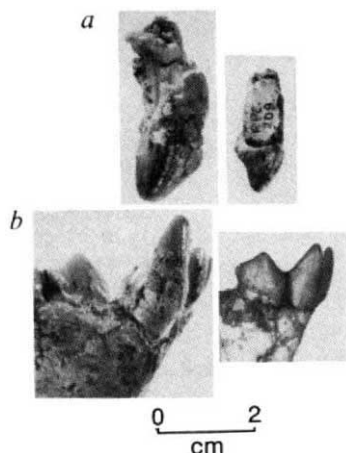


FIG. 2 *Ouranopithecus macedoniensis*. a, Upper canines; left, male (RPI 209); right, female (RPI 208). b, Lower canines; left, male (RPI 75); right, female (RPI 54).

indicating that the posterior part of this bone was steeply inclined. The incisive canal is large, as it is on the specimen RPI 128 (ref. 12) and the whole area is similar to a primitive 'African' naso-maxillary pattern. The palate is deep (12.2 mm at the level of M²), but the maxilla and palatine bones are largely dissolved.

The skull of *Proconsul* differs from XIR-1 by the lack of brow ridge; *Lufengpithecus* has an 'Asian' naso-maxillary pattern (orang pattern)¹³; *Sivapithecus* has the same 'Asian' pattern, high and oval orbits and small inter-orbital region and looks like *Pongo* (Pongidae). In the cranial structures *Ouranopithecus* shows most of the primitive characters of the great apes and Homininae but the brow ridge can be a derived character of African great apes and Homininae. The trend to reduced canines, the absence of the honing wear facet (for upper canine) on the anterior face of the lower third premolar and the round and swollen molar cusps of the molars¹⁴, are shared by *Australopithecus* and *Homo* (Homininae) and all are interpreted as derived characters. The proportions of the lower dentition would demonstrate also that *Ouranopithecus* is linked with Plio-pleistocene Homininae¹⁵. If *Ouranopithecus* is a sister-group of all recent Homininae, we must admit a reversion of these characters for gorillas and chimpanzees. It is more parsimonious to accept *Ouranopithecus* as the sister-group of only the Plio-pleistocene Homininae. The solution which considers *Ouranopithecus* as the sister-group of all great apes and man (Pongidae and Hominidae) would be even less parsimonious. In this case, the polarities of the characters, stated from out-group comparisons into other Catarrhini and from observations of the oldest known hominoids (that is *Proconsul*) are indicated in Table 2. We think it more probable that these features are shared derived features of both *Ouranopithecus* and *Australopithecus afarensis* than homoplasies.

Molecular data have recently provided information on the branching times of the different lineages of hominoids. The branching between man and African apes has been estimated to be 0.5–12 Myr ago^{16,17}, and between pongids and hominids, 10–19 Myr ago^{18,19}. Recently some fossils which bear some characters of Catarrhini (Old World monkeys, apes and man) have been unearthed from marine and fluvial deposits of North Africa which are considered late Eocene (40–45 Myr) on the basis of marine faunas²⁰. This discovery indicates that the Catarrhini are older than previously thought and that the branching times of Primate lineages need to be reappraised. If *Ouranopithecus* is really a hominine, the branching with African great apes may be as old as 12 Myr. We believe that this genus

can be considered a good Miocene ancestral morphotype for *Australopithecus* and man. □

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Endemism centres, refugia and botanical collection density in Brazilian Amazonia

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HERBARIUM specimen collecting in the Brazilian Amazon has been concentrated in widely scattered collecting centres associated with some proposed centres of substrate-independent endemism^{1–3}, suggesting that these may be sampling artefacts. Furthermore, many Amazonian plant species are uncommon, so the more intensely a local flora is studied, the more it will seem to be unique. This weakens the botanical argument for a dry Pleistocene Amazon⁴ and the associated forest refuge theory for the origin of Amazonian plant diversity^{1–3}, because modern endemism centres are used as evidence to define past isolated forest patches—sites of allopatric speciation in a supposedly dry climate (Fig. 1). With a detailed map of botanical collection density, it is possible to recognize the true concentrations of plant endemism, which is important for selecting priority conservation areas to guarantee preservation of unique species.

We analysed the quality and quantity of data available for defining plant species distributions using two approaches: quantifying commonness versus rarity within the herbarium, and mapping the number of collections made from each of the 466 degree-grid squares in the Brazilian Amazon.

The 1,971 specimens of Brazilian Amazonian Chrysobalanaceae in the INPA (Manaus) herbarium in 1984 represented 156 species, of which 32 (20.5%) were collected only once and 105 (67%) were collected less than ten times. When plotted as a ranked histogram (Fig. 2), these data make a striking impression. Despite the fact that experienced collectors and specialists discriminate strongly in favour of rarities, most species remain poorly collected. About 40% of all collections of Brazilian Amazonian angiosperms are in the INPA herbarium, so the shape of this histogram would not be much altered by including data from other herbaria. Chrysobalanaceae

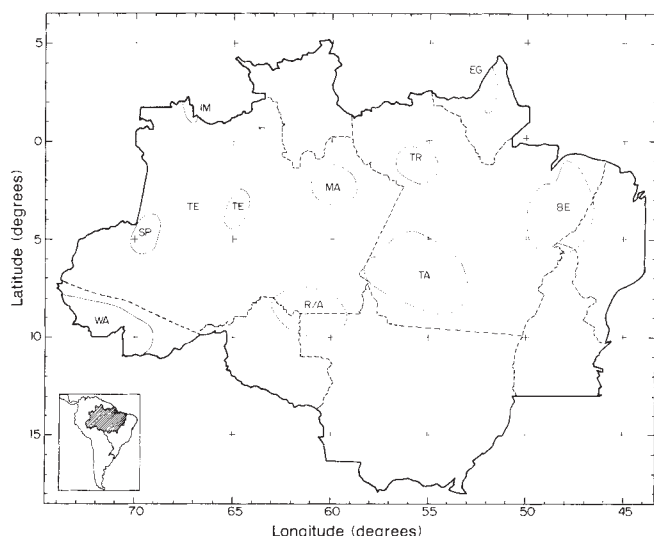


FIG. 1 Ten endemism centres, or Pleistocene refugia, postulated for Brazilian Amazonia based on study of woody angiosperms. Redrawn from most recent and accurate map published² (G. T. Prance, personal communication). From west to east: Western Acre (WA), São Paulo de Olivença (SP), Tefé (TE), Imeri (IM), Rondônia-Aripuanã (R/A), Manaus (MA), Tapajós (TA), Trombetas (TR), East Guiana (EG) and Belém (BE). Tefé endemism centre was erroneously published somewhat west of the town of the same name and is drawn correctly here. Both positions are indicated with same abbreviation 'TE'.

is one of the two large families originally used as evidence for delimiting refugia in the Brazilian Amazon¹. With so many poorly collected species, some presumed local endemics and disjuncts may actually have a wider distribution.

The widespread and commonly collected tree genus *Inga* was selected as a sample for mapping collection density for the nine herbaria holding significant numbers of Brazilian Amazonian angiosperms. Degree-grid squares were determined for 2,779 different *Inga* collections from the five million square kilometres of Brazilian 'Legal' Amazon. Of these, 1,164 were from the INPA herbarium, representing 1.0% of INPA's vascular plant specimens from the target area in January 1989. A map with collection density isolines was developed for all 2,345 plants collected before 1980 (Fig. 3) (later collections were not included when botanical evidence for Amazonian endemism centres was recently considered^{2,3}). This map was then treated (Fig. 4) by putting to zero all degree-grid squares fitting any of six criteria making them unlikely to have been covered by dense dryland evergreen forest during a drier Pleistocene.

For the untreated data, *Inga* collection density per degree square varies from zero to 320 (the total flora would range from zero to about 32,000). Previous attempts at quantifying collection density were based on the number of species—not specimens—per degree square for two moderate-sized genera^{3,5} and thus were not nearly sensitive enough to pick up this extreme bias. An ideal sample of the Brazilian flora for phytogeographical study would have an equal number of collections from each degree square. Both the treated and untreated data sets show that botanical study has been concentrated in collecting centres. The treated set exaggerates the isolation of these centres somewhat by putting intervening areas to zero.

There is an obvious superposition of collection centres with four postulated endemism centres (vestiges of refugia): Tefé, Manaus, Trombetas and Belém. Two other postulated endemism centres, Tapajós and Rondônia-Aripuanã, also have associated collection centres, but with imperfect correspondence. This may be a result of other constraints on the choice of refuge position and size. For example, the Rondônia-Aripuanã refuge was placed in an extensive tract of unbroken humid forest surrounded on three sides by collection centres also with humid forest,

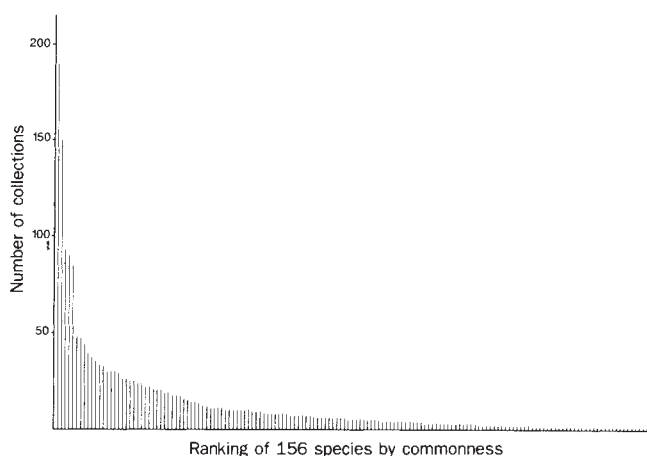


FIG. 2 Commonness and rarity of woody angiosperms in the INPA (Manaus) herbarium, based on all 156 Brazilian Amazonian Chrysobalanaceae in the collection as of 1984. Total sample size is 1,971 specimens.

but close to savanna or drier forest. Under refuge theory, allopatric speciation would occur in the refuge, followed by a slight range expansion as humid forest expanded after the Pleistocene. The theory would attribute endemism in the surrounding collection centres to the refuge. Forests just above the fall line of the Tapajós River were heavily collected by Adolfo Ducke, and this collection centre would furnish apparent endemics and disjuncts attributable to slight range expansion from the Tapajós refuge. The precise configuration of the large Tapajós refuge, however, seems to have been based more on geoscientific evidence⁶ as the bulk of the area within the refuge is botanically unexplored.

Therefore, of the seven refugia (centres of substrate-independent endemism) located entirely within Brazil, only the São Paulo de Olivença refuge is not associated with a collection

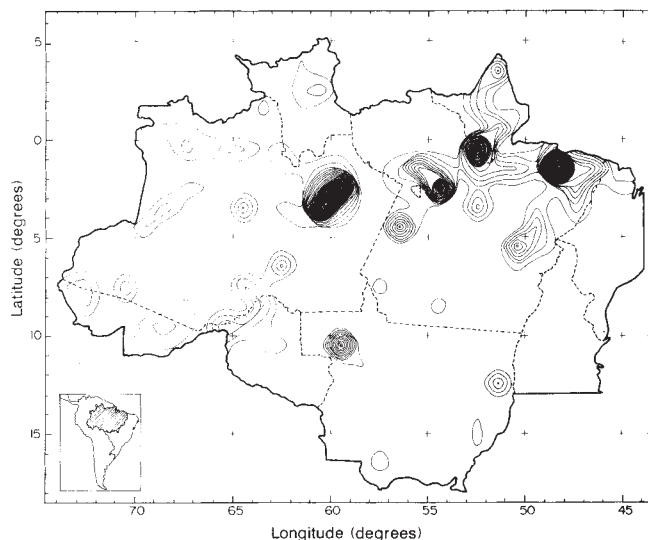


FIG. 3 Density of arborescent angiosperm collections made in Brazilian Amazonia before 1980, based on 1.0% sample from nine herbaria (National Institute for Research in the Amazon at Manaus; Goeldi Museum in Belém; Centre for Humid Tropic Research in Belém; Botanical Garden of Rio de Janeiro; National Museum in Rio de Janeiro; São Paulo Botanical Institute; University of Brasília; New York Botanical Garden; Smithsonian Institution in Washington). Isolines represent number of specimens of genus *Inga* per degree-grid square, plotted at density intervals of five. Squares with fewer than five *Inga* do not appear on map. Density ranges from zero to 320 specimens, with peaks of 160 and 320 over Manaus and Belém, respectively. Duplicate collections deleted, leaving total sample of 2,345 specimens.

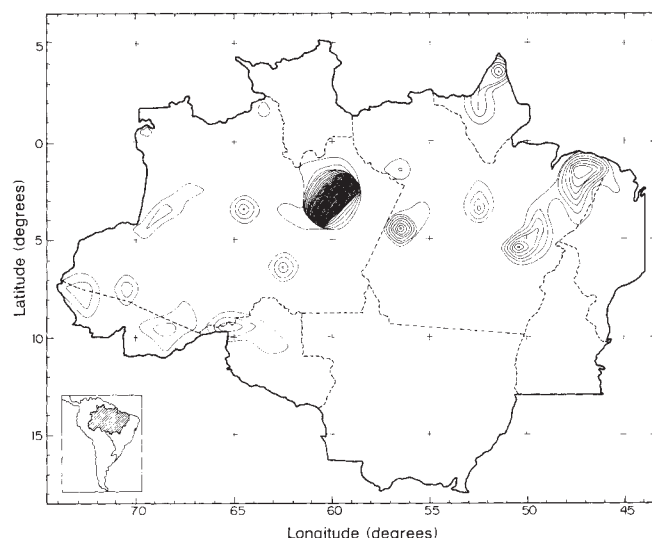


FIG. 4 Same data as shown in Fig. 3 treated by putting to zero all degree-grid squares where collections were known to be primarily from areas unlikely to have been forested during a drier Pleistocene, that is, savanna areas, transition forest, high forest close to savanna or transition forest (such as at Jari, Aripuanã and Obidos), white sand areas, sandstone or canga formation and floodplain or low-lying *terra firme* areas downstream of Manaus, probably inundated during previous Pleistocene interglacials.

centre and not suspected of being an artefact. Of the three refugia on international borders, the East Guiana refuge is heavily collected in its Brazilian portion. The other two are based largely on collections from outside Brazil.

Our analysis does not weaken the arguments for endemism centres and refugia based on the distribution of birds^{7,8}, butterflies^{9,10} and lizards¹¹, although in the case of birds, at least, the data are subject to alternative interpretations¹². With the collecting density map presented here it may now be possible for phytogeographers and conservation planners interested in identifying endemism centres to correct for the strong bias in the botanical sample. Centres of diversity determined by superimposing the known ranges of hundreds of species should incorporate similar corrections. As the phytogeographical data base is highly biased towards collection centres, conservation planners aiming to preserve unique plant communities should give greater weight to more equitably distributed co-variate data. These include maps of climate, geology and soil, as well as remote sensing of geomorphology and vegetation physiognomy coupled with rapid standardized field inventories of forest diversity, structure and composition. Our map clearly indicates the most critical area for future botanical collecting efforts: deforestation in the Brazilian Amazon is concentrated in southern Pará and northern Mato Grosso, a large, botanically unexplored area. □

Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala

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RECEPTORS for *N*-methyl-D-aspartate (NMDA) seem to have a critical role in synaptic plasticity¹⁻⁴. NMDA antagonists (such as AP5) prevent induction of long-term potentiation^{5,6}, an activity-dependent enhancement of synaptic efficacy mediated by neural mechanisms that might also underlie learning and memory. They also attenuate memory formation in several behavioural tasks⁷⁻¹⁷; there are few data, however, implicating an NMDA-sensitive measure of conditioning based on local infusion of antagonists into a brain area tightly coupled to the behavioural response used to assess conditioning. We now show that NMDA antagonists infused into the amygdala block the acquisition, but not the expression, of fear conditioning measured with a behavioural assay mediated by a defined neural circuit (fear-potentiation of the acoustic startle reflex). This effect showed anatomical and pharmacological specificity, and was not attributable to reduced salience of the stimuli of light or shock used in training. The data indicate that an NMDA-dependent process in the amygdala subserves associative fear conditioning.

The role of NMDA receptors in the amygdala in associative fear conditioning was assessed using fear-potentiation of acoustic startle, a simple reflex mediated by a defined neural pathway contained in the brainstem and spinal cord¹⁸. The central nucleus of the amygdala projects directly to one of the brainstem nuclei critical for startle¹⁹ and lesions of this amygdaloid nucleus, or interruption of the pathway connecting the amygdala to the startle circuit, block the ability of conditioned or unconditioned fear stimuli to elevate startle^{20,21}. Conversely, weak electrical stimulation of the amygdala markedly increases startle amplitude²². The lateral and basolateral amygdaloid nuclei, which project directly to the central nucleus, contain high densities of NMDA receptors²³ and lesions of these nuclei markedly attenuate the acquisition of fear-potentiated startle (C.B.S. and M.D., unpublished observations). If an NMDA-dependent process in the amygdala is involved in associative fear conditioning, then such conditioning should be sensitive to manipulations of local NMDA receptor function.

To test this, 132 rats were implanted with bilateral cannulae aimed at the basolateral amygdaloid nuclei. Thirteen control animals were implanted with cannulae aimed at the interpositus nuclei of the cerebellum, an area involved in classical conditioning of motor responses, but not in fear conditioning²⁴. One week after surgery, animals were bilaterally infused on two successive days with 0.5 µl of one of the following: vehicle (artificial cerebrospinal fluid (ACSF) or 0.1 M phosphate buffer, $n = 23$); DL-2-amino-5-phosphonopentanoic acid (AP5; 6.25 ($n = 5$), 12.5 ($n = 9$), 25 ($n = 13$) or 50 nmol ($n = 5$), DL-2-amino-7-phosphonoheptanoic acid (AP7, 50 nmol ($n = 6$); ACSF ($n = 5$) or propranolol (40 nmol ($n = 7$); ACSF ($n = 7$)). Solutions were adjusted to pH 7.0. Five minutes later, animals were presented with 10 light-footshock pairings at 4-6-min intervals. Conditioning was assessed one week later by comparing startle elicited by a noise burst presented alone, with startle elicited by a noise burst in the presence of the light previously paired with footshock. Magnitude of conditioned fear was defined as the increase in startle amplitude on the light-noise versus noise-alone trials.

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Infusion of AP5 or AP7 into the amygdala blocked the acquisition of fear-potentiated startle relative to vehicle controls (Fig. 1a, b). But, the β -adrenergic antagonist propranolol, which attenuates one-trial inhibitory avoidance conditioning when infused into the amygdala after training²⁵ and has powerful local anaesthetic effects²⁶, did not attenuate the acquisition of fear-potentiation (Fig. 1c). No motor impairment (such as catalepsy or ataxia) was observed after local infusion of any of these doses of AP5 into the amygdala. Bilateral local AP5 (100 nmol) administration into the interpositus nucleus of the cerebellum before training ($n=8$) did not affect fear-potentiation of startle, even at a dose eight times that which blocked conditioning in the amygdala (Fig. 2a). In addition, animals infused with AP5 5 days after training, but 1 week before testing ($n=8$), did not differ at testing from vehicle controls ($n=5$; Fig. 2b), indicating that the observed block of acquisition by AP5 did not result from any permanent damage to the amygdala that might have developed over the 1-week infusion-test interval. Moreover, animals with AP5 (50 nmol) infused into the amygdala reacted as vigorously as controls to the 0.6 mA footshock used in training (Fig. 2c). This footshock-induced increase in activity has previously been shown to be markedly reduced by

lesions of the amygdala²¹ or systemic injection of morphine (J. M. Hitchcock and M. Davis, unpublished observations). This suggests that AP5 does not block the acquisition of fear conditioning by producing analgesia or by decreasing the ability of the footshock to activate the amygdala.

To test whether a smaller amount of drug would block conditioning, a shorter training session was used consisting of five light-shock pairings presented at a 2-min interval. Under these conditions, infusion of AP5 5 min before training blocked the acquisition of potentiated startle in a dose-dependent manner and significantly attenuated fear conditioning at one-fourth of the dose required to block conditioning using the original, longer training procedure (Fig. 3a; ACSF ($n=5$); AP5 1.6 ($n=5$); 3.12 ($n=6$); 6.25 ($n=6$); or 12.5 nmol ($n=6$)). Importantly, however, even the highest effective dose of AP5 did not block the expression of fear-potentiated startle in animals trained in the absence of the drug (Fig. 3b; ACSF ($n=5$); AP5 12.5 nmol ($n=6$)). This strongly suggests that local infusion of AP5 into the amygdala does not prevent synaptic transmission of visual information to the amygdala.

Histological examination of cannula placements in a subset of animals ($n=20$) suggests that the compounds used in this

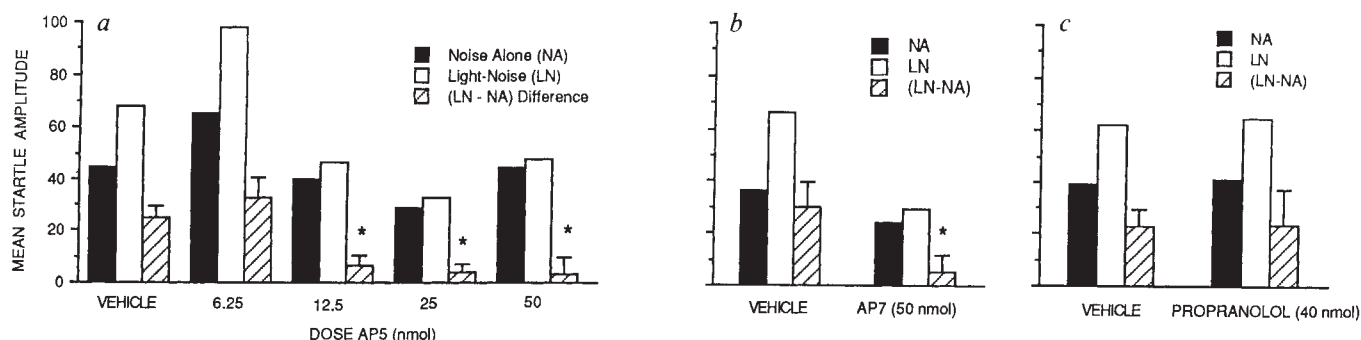


FIG. 1 NMDA-specific antagonists infused into the basolateral nuclei of the amygdala immediately before light-shock training sessions blocked the acquisition of conditioned fear measured with potentiated startle tested 1 week later. **a**, AP5 caused a dose-dependent decrease in conditioned fear-potentiation of startle ($F_{(1,50)}(1.50)=14.75$, $P<0.001$), with significant attenuation compared with vehicle at doses of ≥ 12.5 nmol ($P<0.05$, Newman-Keuls). **b**, AP7 also blocked fear-potentiated startle ($t(9)=2.23$, $P<$

0.05). But rats receiving bilateral infusion of propranolol **c**; (40 nmol) into the amygdala at training showed significant fear-potentiation of startle, ($t(6)=2.92$, $P<0.03$) and did not differ from vehicle control animals at testing ($t(12)=1.32$, not significant). The apparatus and potentiated startle training and testing procedures have been described previously^{28,29}. The standard potentiated startle paradigm require two 45-min training sessions.

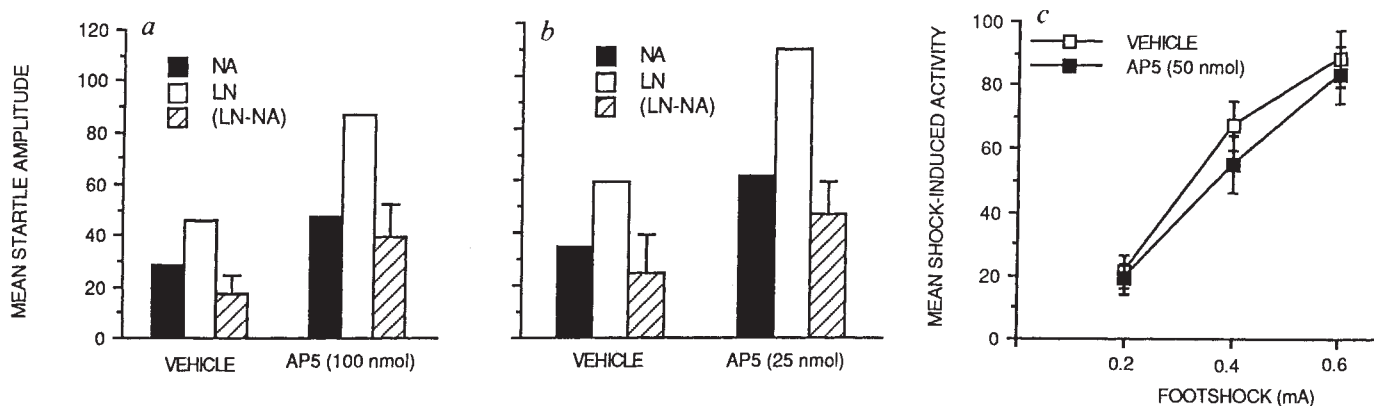


FIG. 2 **a**, Infusion of a high dose of AP5 (100 nmol) into the cerebellar interpositus nuclei at training did not block or attenuate fear-potentiation of acoustic startle ($t(12)=1.43$, not significant). **b**, Startle amplitude at testing was not attenuated as a result of AP5 (25 nmol) infusion into the amygdala, on two successive days, 5 days after standard potentiated startle training ($t(11)=1.21$, not significant). As in the previous experiments, the

infusion-testing interval was 1 week. **c**, Reactivity to footshock was not different for animals given AP5 (50 nmol ($n=10$)) or vehicle ($n=10$) into the amygdala ($F(1,18)=0.84$, not significant). During shock-activity testing, rats received ten 500 ms shocks at 1-s intervals at each of the following intensities: 0.2, 0.4 and 0.6 mA, presented in ascending order. Activity was assessed by cage displacement for the 500 ms period after shock onset.

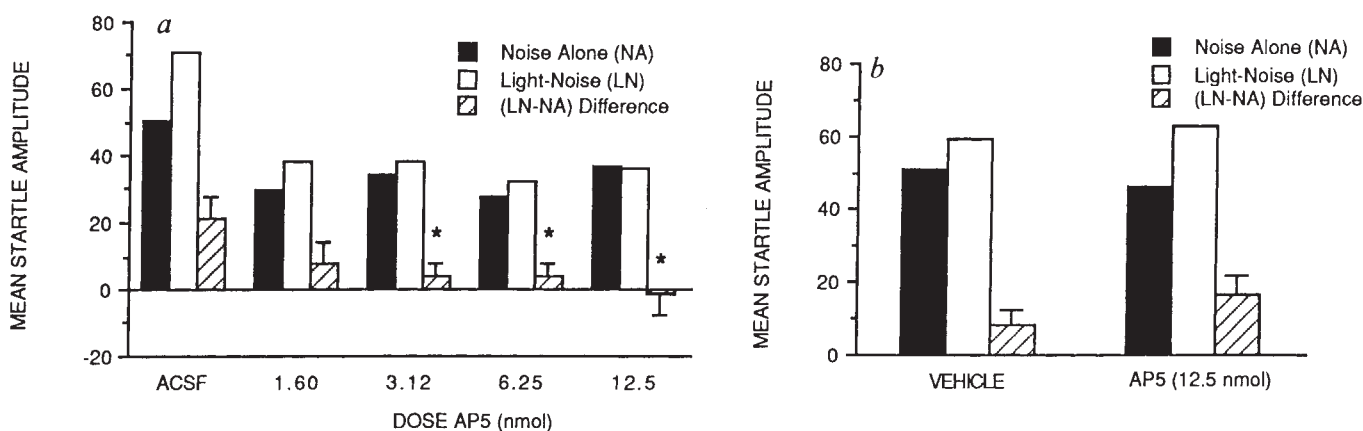


FIG. 3 *a*, Infusion of AP5 into the amygdala immediately before a shorter training procedure (a 15-min session consisting of five light (conditioned stimulus)-footshock (unconditioned stimulus) pairings, with a 2-min inter-stimulus interval) dose-dependently blocked acquisition of conditioned fear ($F_{\text{linear}}(1, 23) = 7.43, P < 0.01$), and significantly reduced potentiated startle at doses of 3.12 nmol and greater. *b*, The highest effective dose of AP5 (12.5 nmol) used to block acquisition of conditioned fear in the short training

study attenuated potentiated startle through actions on a substrate ≤ 1.0 mm from the basolateral nucleus. Therefore, two rats with one or both cannula tips located beyond that distance did not show a decrease in fear conditioning, even when normally effective doses were infused.

The finding that NMDA receptor antagonists infused into the amygdala blocked fear-potentiated startle indicates that an NMDA-mediated process at that level is critical for the acquisition of conditioned fear. The many demonstrations that NMDA antagonists also block induction of long-term potentiation (LTP), and the finding in intracellular studies that LTP occurs in the amygdala²⁷, suggest that an NMDA-dependent form of LTP in the amygdala might underlie fear conditioning.

Investigations of the physiological and biochemical mechanisms of learning and memory have mainly made use of *in vitro* preparations. The results of the present study, suggesting the existence of an NMDA-dependent process in the amygdala subserving fear conditioning, show that it may now be possible to use a behavioural measure, with a defined neural circuit tightly coupled to the possible site of plasticity, to evaluate the role of these mechanisms *in vivo*. □

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procedure did not attenuate the expression of fear-potentiated startle, compared with vehicle controls, when infused into the amygdala immediately before testing. Hence the overall difference between the light-noise and noise-alone trials was statistically significant ($F(1, 9) = 11.97, P < 0.01$) but there was no group by trial-type interaction ($F(1, 9) = 1.18$, not significant) and significant potentiated startle occurred in the AP5 group ($t(5) = 2.91, P < 0.03$).

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Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation

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IN both vertebrates and invertebrates, long-term memory differs from short-term in requiring protein synthesis during training^{1,2}. Studies of the gill and siphon withdrawal reflex in *Aplysia* indicate that similar requirements can be demonstrated at the level of sensory and motor neurons which may participate in memory storage. A single application of serotonin³, a transmitter that mediates sensitization, to individual sensory and motor cells in dissociated cell cultures leads to enhanced transmitter release from the sensory neurons that is independent of new macromolecular synthesis. Five applications of serotonin cause a long-term enhancement, lasting one or more days, which requires translation and transcription^{2,3}. Prolonged application or intracellular injection into the sensory neuron of cyclic AMP, a second messenger for the action of serotonin, also produce long-term increases in synaptic strength^{4,5}, suggesting that some of the gene products important for long-term facilitation are cAMP-inducible. In eukaryotic cells, most cAMP-inducible genes so far studied are activated by the cAMP-dependent protein kinase (A kinase), which

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phosphorylates transcription factors that bind the cAMP-responsive element TGACGTCA. The cAMP-responsive element (CRE) binds a protein dimer of relative molecular mass 43,000, the CRE-binding protein (CREBP), which has been purified and shown to increase transcription when phosphorylated by the A kinase⁶⁻¹¹. Here we show that extracts of the *Aplysia* central nervous system and extracts of sensory neurons contain a set of proteins, including one with properties similar to mammalian CREBPs, that specifically bind the mammalian CRE sequence. Microinjection of the CRE sequence into the nucleus of a sensory neuron selectively blocks the serotonin-induced long-term increase in synaptic strength, without affecting short-term facilitation. Taken together, these observations suggest that one or more CREB-like transcriptional activators are required for long-term facilitation.

We first determined whether the *Aplysia* central nervous system (CNS) contains a CREBP-like protein. Gel mobility shift

assays on extracts of *Aplysia* CNS using a rat somatostatin CRE probe, gave three specifically retarded bands (Fig. 1a). The same number of retarded bands was seen when the CRE sequence from the vasoactive intestinal protein (VIP) gene was used as the probe (data not shown). A similar mobility shift pattern was also observed with extracts from *Aplysia* sensory neurons. Moreover, using antibody to the mouse CREBP¹², we have found cross-reactivity with a protein of relative molecular mass 43,000 (43K) from *Aplysia* CNS extract which is highly enriched after partial purification on a CRE affinity column (data, including a western blot, not shown).

Binding of *Aplysia* proteins to somatostatin CRE is sequence-specific. It is effectively inhibited to varying degrees by non-radioactive oligonucleotides containing the CRE sequences from somatostatin, VIP, *fos* or enkephalin (Fig. 1a). The differential inhibition seen may reflect either differences in the affinity of the protein for slightly different CRE sequences, or the contribution to binding affinity of the distinctive CRE flanking sequences^{13,14}. By contrast, nonspecific linear plasmid DNA (PKSM13⁺) did not compete with binding, and only at a very high concentration (Fig. 1a) was there some nonspecific decrease in binding. Two mutant oligonucleotides (TGAAGCCA and CTTAAGTG) with the same flanking sequences as the somatostatin probe also failed to compete with the binding. Moreover, the two AP-1 sequences (TGAGTCA) differing from the CRE sequence in only one nucleotide compete weakly at the concentrations we used (Fig. 1b). Similar weak competition has been previously reported^{8,14-16}.

To characterize the DNA-protein interaction at the CRE site, we carried out DNase I footprinting in parallel nuclear extracts derived from *Aplysia* CNS and HeLa cells. Addition of HeLa nuclear extract gave two specific retarded bands and one non-specific band in the gel shift assay (data not shown). Both the specific retarded bands are protected from DNase I digestion at and around the CRE sequence as are the three retarded bands of *Aplysia* CNS extract (Fig. 2). Comparison of the two hypersensitive sites shows that the DNase protection by HeLa extract covers more nucleotides than the *Aplysia* extract.

Next we determined whether a protein like the CREBP is involved in long-term facilitation, which is transcription dependent and initiated by cAMP. As the site of plasticity for this long-term increase in transmitter release resides within the sensory neuron¹⁷, we isolated sensory and motor neuron pairs in culture¹⁸, and injected double-stranded somatostatin CRE sequence into the nucleus of the sensory neuron (Fig. 3). After injection, we treated the culture dish with five exposures of serotonin and monitored long-term facilitation by examining the connection 24 h later. If the injected CRE sequence competed for binding of a CREBP-like protein, this protein might be prevented from activating the cAMP-inducible genes, despite the increase in cAMP produced by serotonin. A blockade of long-term facilitation might result (Fig. 3b).

We found that cells injected with control (mutant) oligonucleotides showed an increase in synaptic strength one day after serotonin treatment ($31\% \pm 14\%$ s.e.m., $n = 7$), which was comparable to that occurring in uninjected cells ($38\% \pm 13\%$ s.e.m., $n = 43$) (Fig. 4a, b). By contrast, cells injected with somatostatin CRE did not show an increase in the excitatory postsynaptic potential (e.p.s.p.) 24 h after serotonin treatment and were comparable to cells not exposed to serotonin and significantly different from control cells exposed to serotonin ($7\% \pm 8.5\%$ s.e.m., $n = 19$, $P < 0.05$). Whereas the CRE oligonucleotides blocked long-term facilitation, these oligonucleotides had no effect on short-term facilitation, as tested either immediately after injection (data not shown) or 24 h later (Fig. 4c). Moreover, the effect on long-term facilitation was titratable. Injection of 20-fold less CRE blocked long-term facilitation only partially ($n = 4$).

To rule out a nonspecific reduction in the long-term facilitation attributable to a general effect on transcription, due to either

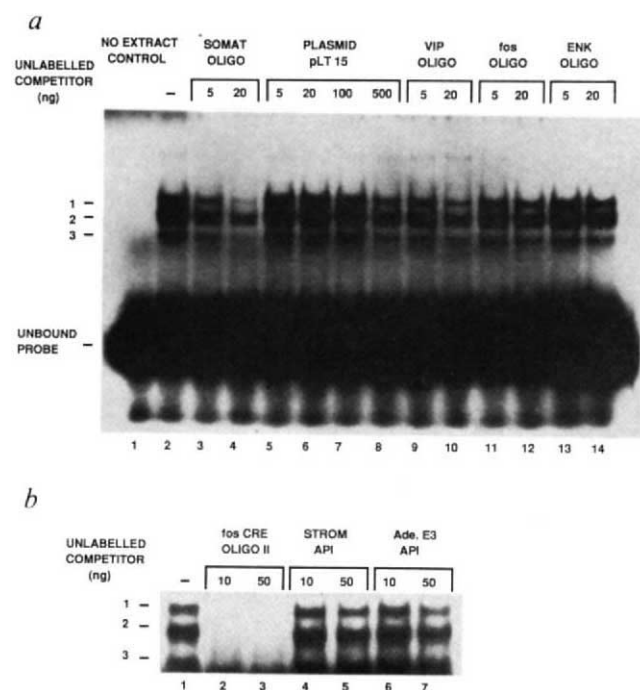


FIG. 1 Specific binding of *Aplysia* CNS extract to CRE of rat somatostatin gene. *a*, Protein binding to CRE was performed by gel-retardation assay. The positions of the free and of the three retarded bands are shown. The probe, and the probe plus extract in the absence of competitor are in lanes 1, 2. The three retarded bands can be competed out specifically by non-radioactive somatostatin CRE (lanes 3, 4) but not by linear nonspecific plasmid DNA (lanes 5-8). The competition by CRE sequence from VIP (-99 to -58), *fos* (-330 to -276), and enkephalin (-114 to -70) is shown in lanes 9-14 (ref. 9). *b*, AP-1 sequence does not compete with the binding of the protein to CRE. Lane 1 shows the binding of the *Aplysia* CNS extract to somatostatin CRE in the absence of competitor. The second CRE sequence was from *fos* gene (-78 to -48) (ref. 23) competes well with binding (lanes 2, 3). But the AP1 sequence from rat stromolysin (strom) (-84 to -57) (ref. 24) or the adenovirus E3 gene (Ade. E3) (-109 to -79) (ref. 17) does not compete to the same extent as the CRE sequences at the concentrations tested (lanes 4-7).

METHODS. *Aplysia* CNS (abdominal, pleural and pedal ganglia) from 100-120 g animals were dissected from isotonic $MgCl_2$ -anaesthetized animals. The total-protein extract from the desheathed ganglia was prepared as described previously²⁵. Two complementary oligonucleotides corresponding to the rat somatostatin CRE (-75 to -26) containing *Bam*H1 and *Pst*I sites were synthesized, hybridized, and cloned into vector PKSM13⁺. The assay probe was prepared by digesting the plasmid with *Eco*RI, filling the ends with radioactive dATP and dTTP, followed by *Bam*H1 digestion and purification on an acrylamide gel. Synthetic oligonucleotides were used for competition assay. The assay was carried out as described previously²⁶ using $\sim 5 \mu g$ protein, ~ 0.2 - 0.5 ng of probe, and $1 \mu g$ each of salmon sperm DNA and poly(dI-dC):poly(dI-dC) as non-specific competitor in a $20 \mu l$ reaction volume.

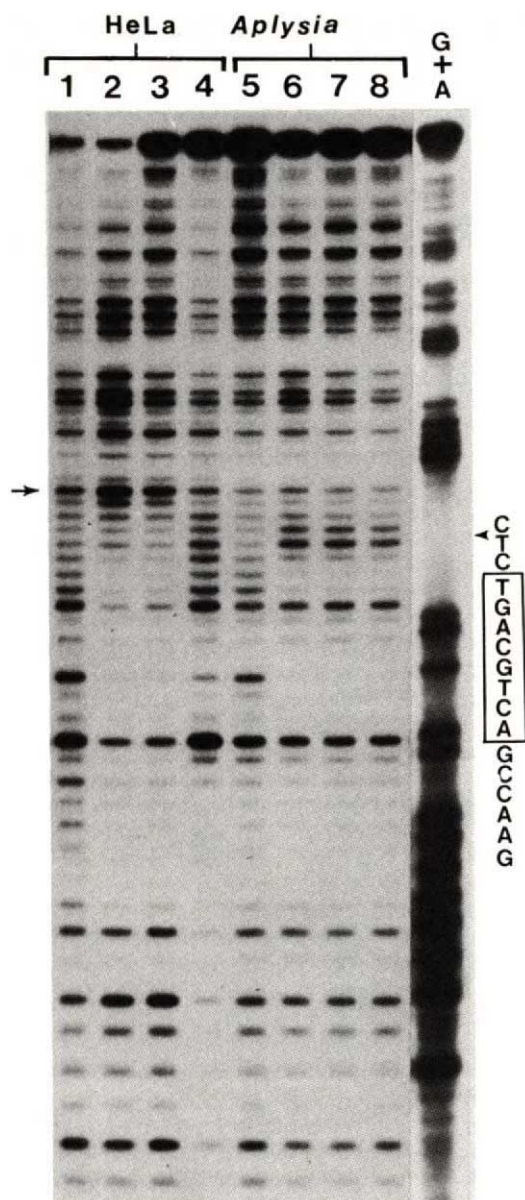


FIG. 2 Analysis of the protein-DNA interaction at the CRE site. DNase I footprinting of the coding strand of the rat somatostatin CRE with HeLa nuclear and *Aplysia* CNS extract. An autoradiograph of the sequencing gel is shown. Two retarded bands were seen using HeLa nuclear extract. The footprint of the unbound (lanes 1), nonspecifically bound (lane 4), and the two retarded bands (lanes 2, 3) are shown. The footprint of the unbound band (lane 5) and three retarded bands (lanes 6–8) with the partially purified heparin-agarose fraction of the *Aplysia* CNS extract is shown. The DNase I hypersensitive sites are shown by arrows. The same probe was independently cleaved with piperidin (A + G ladder) for sequence alignment of the CRE motif and is shown at the right.

METHODS. The footprinting reactions were prepared in a total volume of 50 μ l containing 1–2 ng of end-labelled DNA probe as described previously²⁵. The probe was obtained from PKSM13⁺ plasmid containing the CRE sequence, by digesting with *Xho*I, filling the end with ³²P nucleotides and Klenow polymerase followed by a second digestion at the *Bam*HI site. The fragment containing the CRE sequence was purified in an acrylamide gel. About 20 μ l protein was incubated with 1–2 ng of the probe in the presence of nonspecific competitor for 30 min at room temperature. After DNase I digestion (Pharmacia; 10 units for 30 s), the free and retarded bands were separated on a low-ionic-strength acrylamide gel. The bands were detected by autoradiography, excised and eluted in 0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS overnight at 37 °C. The eluted DNA was run on a 6% sequencing gel.

titration of a general transcription factor(s) or some structural features specific to enhancer sequences, we also injected two other known enhancer sequences: (1) the consensus heat-shock element (HSE); and (2) the NF- κ B enhancers^{19,20}. Neither injection of HSE, whose sequence is conserved from human to yeast, nor injection of NF- κ B enhancer, affected the increase in synaptic strength seen 24 h after serotonin treatment (Fig. 4b). Gel shift assay shows that a heat-shock transcription factor is present in sensory neuron extracts and its binding can be induced by heat treatment.

To ensure that the CRE injection did not simply produce its blockade by a general inhibition of transcription, we injected

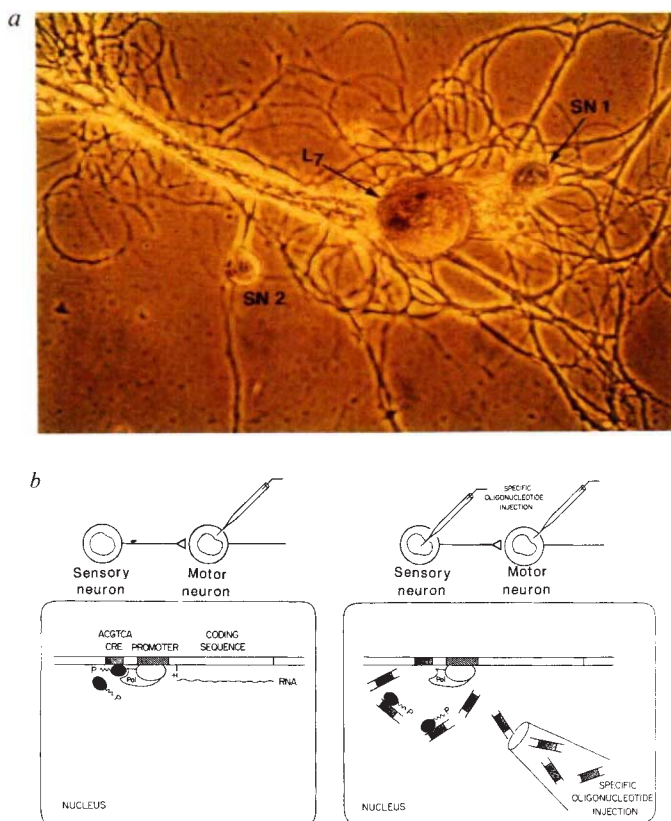
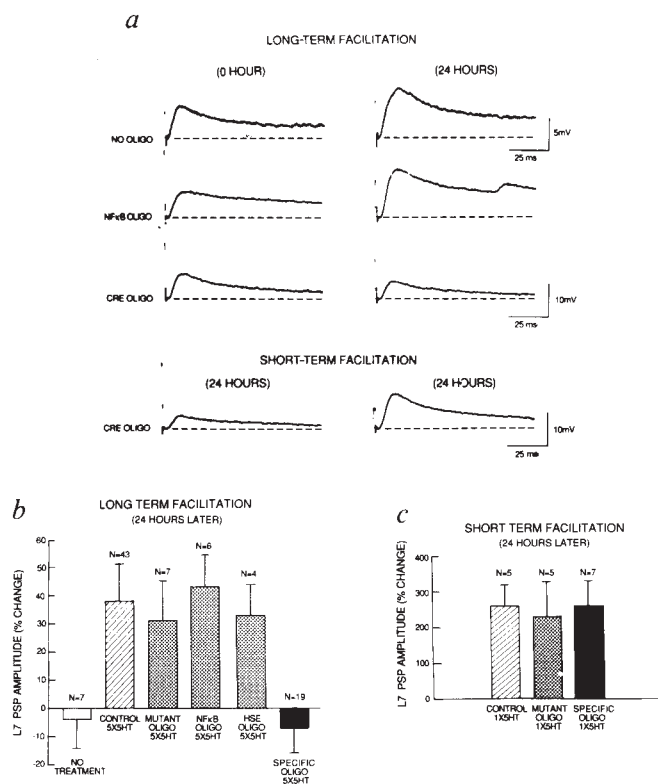


FIG. 3 *Aplysia* sensory-motor neuron culture system used to study the inhibition of long-term facilitation by CRE oligonucleotide injection. *a*, Phase contrast photomicrograph of a culture of motor neuron L7 and two sensory neurons. One of the sensory neurons was injected (SN2) with the sensory oligonucleotide, whereas the other one was uninjected or injected with a control oligonucleotide. *b*, A schematic representation of the experimental design to study the effect of CRE injection.

METHODS. One to three pleural sensory neurons from a 100 g animal and an L7 motor neuron from a juvenile animal were co-cultured as described previously^{18,27}. After 4–5 days in culture, the amplitude of the e.p.s.p. evoked in L7 by each sensory neuron was measured using extracellular electrodes to stimulate the sensory cell. One of the sensory cells was pressure-injected with 10–30 μ l of 5 μ g ml⁻¹ double-stranded somatostatin CRE oligonucleotides (about 10⁶ copies) in diethylpyrocarbonate-treated 0.5 M potassium acetate, 10 mM Tris HCl (pH 7.6) and 0.2% Fast Green. The other cell was either injected with control oligonucleotide or was left uninjected. After recording, the cultures were treated with 5 μ M serotonin to induce long-term facilitation². After 24 h the e.p.s.p. was measured. The nucleotide sequences of oligonucleotides used in these studies were: CRE, 5'-GGCCTCCTTGCT-GACGTCAGAGAGAGAGTTCTGCA-3'; Mutant, 5'-GGCCTCCTTGCTTAAGT-GGAGAGAGAGTTCTGCA-3'; NF- κ B, 5'-GATCTCAACAGAGGGGACTTCCGAG-GCCA-3'; HSE, 5'-GGAGCGCGCCTCGAATGTTCTAGAAAAGGCTGCA-3'. Oligonucleotides were synthesized with a *Pst*I overhang, except NF- κ B which has a GATC overhang. Both the complementary strands were purified and hybridized to each other. After hybridization, the double-stranded oligonucleotides were phenol-extracted and then precipitated with ethanol before use.



the CRE oligonucleotide into the nucleus of the R2 neuron, which is sufficiently large for us to examine the heat-shock response in the same cell. As the volume of R2 is about 100 times greater than that of the sensory neurons, we injected a correspondingly greater amount of oligonucleotide. Induction of the family of heat-shock proteins was not affected, suggesting that the CRE oligonucleotide injection does not cause a general inhibition of transcription. (Similarly, injection of digested PKSM13⁺ plasmid DNA (average size 128 nucleotides), or of the vehicle, failed to block the increase in synaptic strength in the sensory neurons.)

Our results, based on mobility shift and western blot assays, indicate that the nervous system of *Aplysia* contains a CREBP-like protein of relative molecular mass 43K. Moreover, the selective blockade of long- but not short-term facilitation by the binding of the CREBP, in a sequence-specific manner, implicates CREBP as one component of the molecular machinery for setting up long-term facilitation. Although the sensory neuron-motor neuron system still precludes a direct examination of the mechanism by which binding of CREBP blocks long-term facilitation, titration of CREBP by excess CRE in 3T3 cells blocks induction of cAMP-inducible genes²¹. We therefore think it likely that the CRE oligonucleotide works by inhibiting the action of CREBP, either on cAMP-inducible genes or on constitutively expressed genes that encode proteins that turn over rapidly²². It is also possible that the oligonucleotide works by titrating a general transcription factor that binds to CREBP, however we think this is less likely as injection of the HSE oligonucleotide does not block long-term facilitation.

These experiments also provide additional support (see refs 4, 5) for the importance of the cAMP pathway in initiating long-term facilitation. The data do not exclude, however, the possibility that other second messenger systems also participate in the long-term process. Indeed, we and others have found that CREBP can be phosphorylated by the C kinase, by calcium-calmodulin-dependent protein kinase II, and by casein kinase II (unpublished results) as well as by the A kinase. Moreover, CREBP is probably not the only protein factor important for the induction of the long-term process.

FIG. 4 Injection of CRE oligonucleotides blocks serotonin-induced long-term facilitation. **a**, The e.p.s.p. on to the L7 motor neuron at 0 h (before serotonin treatment) is compared with the first e.p.s.p. 24 h after the treatment. Injection of the control oligonucleotide does not affect the increase in the e.p.s.p. whereas CRE injection blocks the increase 24 h after treatment. Short-term facilitation in the presence of serotonin is not affected by CRE oligonucleotide injection 24 h after the long-term training procedure. **b**, Summary of the blockade of the serotonin-induced increase in long-term facilitation by CRE injection. The height of each bar is the percentage change in the e.p.s.p. amplitude $\pm$ s.e.m. re-tested 24 h after treatment. (A two-tailed *t*-test comparison of means indicated that the decrease in e.p.s.p. in cultures injected with CRE oligonucleotide is significantly different ($P < 0.05$) from the increase in e.p.s.p. in the cells injected with either the mutant or NF- κ B oligonucleotides.) **c**, Summary of the pooled data for short-term facilitation 24 h after injection. In contrast to long-term facilitation, the serotonin (5 μ M) was applied after the e.p.s.p. was first depressed. Five stimuli were given with an interstimulus interval of 30 s, and this resulted in 70–80% depression in e.p.s.p. amplitude. This serotonin now produced an increase in e.p.s.p. amplitude by the seventh stimulus. The increase in short-term facilitation was measured by calculating the percentage increase in the seventh e.p.s.p. amplitude as compared with the fifth e.p.s.p. amplitude. Because the facilitation here was of a depressed e.p.s.p., the percentage facilitation is larger than the long term, where only the non-depressed e.p.s.p. was examined.

METHODS. Standard electrophysiological techniques were used for recording from the cultured neurons². During each recording session, the motor cell was impaled with a glass electrode (20–30 M Ω) filled with 3 M potassium acetate. The cells were recorded in a medium of equal volumes of artificial sea water and L15 culture medium (Flow Laboratory). *Aplysia* haemolymph was then added to a final concentration of 5% and the medium was filter sterilized.

Nonetheless, as binding of CREBP seems to be an early step in long-term facilitation, and is a target for both cAMP-dependent and other kinases, purification of CREBP from *Aplysia* and characterization of the upstream region to which CREBP might bind in genes induced by serotonin should allow further study of the causal steps involved in the induction of the long-term process. Moreover, the approach used here to block gene function by intranuclear oligonucleotide injection might be generally useful in studying gene action in nerve cells. $\square$

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Direct evidence for the contribution of the unique I-A^{NOD} to the development of insulinitis in non-obese diabetic mice

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INSULIN-dependent diabetes mellitus is characterized by the infiltration of lymphocytes into the islets of Langerhans of the pancreas (insulinitis) followed by destruction of insulin-secreting β -cells leading to overt diabetes¹⁻⁵. The best model for the disease is the non-obese diabetic (NOD) mouse^{6,7}. Two unusual features of the class II major histocompatibility complex (MHC)^{8,9} of the NOD mouse are the absence of I-E⁹ and the presence of unique I-A molecules (I-A^{NOD})¹⁰, in which aspartic acid at position 57 of the β -chain is replaced by serine. This feature is also found in the HLA-DQ chain of many Caucasians with insulin-dependent diabetes mellitus¹¹⁻¹³. We have previously reported that the expression of I-E prevents the development of insulinitis in NOD mouse^{14,15}. Here we report that the expression of I-A^k ($A\alpha^k A\beta^k$) in transgenic NOD mice can also prevent insulinitis, and that this protection is seen not only when the I-A β -chain has aspartic acid as residue 57, but also when this residue is serine. These results

show that the single amino-acid substitution at position 57 of the I-A β -chain from aspartic acid to serine is not sufficient for the development of the disease.

Eight lines of NOD mice transgenic with wild-type I-A^k β -chain ($A\beta^k$) were established by microinjection of the ~21 kilobase pair (kb) *SalI* fragment containing the entire $A\beta^k$ gene into fertilized eggs of NOD mice¹⁵⁻¹⁷. Two lines of $A\alpha^k$ transgenic NOD mice were obtained by microinjection of the ~11 kb *SmaI-SalI* fragment containing the entire $A\alpha^k$ gene. In addition, three lines of NOD mice transgenic with a mutant $A\beta^k$ in which Asp 57 is replaced by Ser 57 ($A\beta^k$:57Ser transgenic mice) were also obtained. The $A\beta^k$:57Ser gene was derived by *in vitro* mutagenesis¹⁸ using mutagenic primer, in which the coding sequence for Asp at position 57 (GAC) was replaced with that for Ser (TCC). We obtained three lines of $A\alpha^k A\beta^k$ and three lines of $A\alpha^k A\beta^k$:57Ser transgenic mice by breeding $A\alpha^k$ transgenic mice with $A\beta^k$ or $A\beta^k$:57Ser transgenic mice.

The expression of transgenic products was analysed by surface staining of peripheral blood lymphocytes (PBL) with monoclonal antibody 40F for $A\beta^k$ or $A\beta^k$:57Ser chain (Fig. 1A). In $A\beta^k$ transgenic mice (Fig. 1Ac), the level of $A\alpha^d A\beta^k$ (I-A^{d/k}) expression was less than 50% of that of I-A^{k/k} (I-A^k) in a control C3H mouse (Fig. 1Ab). But in almost all $A\alpha^k A\beta^k$ transgenic mice (Fig. 1Ae), the level of I-A^k expression was more than 100% of that of a C3H mouse. The low level of expression of I-A^{d/k} in $A\beta^k$ transgenic mice may be due to the low pairing efficiency between endogenous $A\alpha^d$ and transgenic $A\beta^k$ chain^{10,19}, because the level of $A\beta^k$ transcript was about the same both in $A\beta^k$ and $A\alpha^k A\beta^k$ transgenic mice (Fig. 2a). In $A\alpha^k A\beta^k$:57Ser transgenic mice (Fig. 1Af), the level of I-A^{k/k}:57Ser (I-A^k:57Ser) expression was around 100% of the level of I-A^k in a C3H mouse. The presence of mutant messenger RNA was confirmed by northern blot analysis using mutagenized oligonucleotide probe which had been used also for site directed mutagenesis (Fig. 2b). $A\beta^k$ or $A\beta^k$:57Ser mRNAs were detected in spleen, thymus and lung (Fig. 2). Immunohistochemical analysis revealed a normal pattern of expression of I-A^k and I-A^k:57Ser molecules in the epithelium of medulla

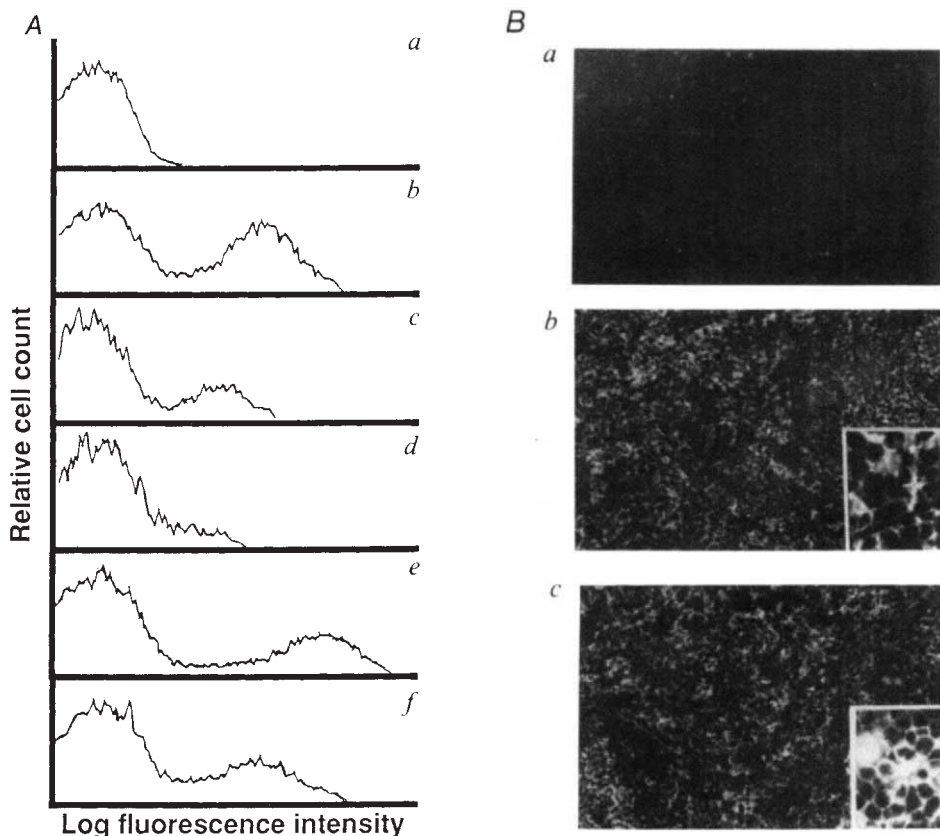


FIG. 1 A, Immunofluorescent analysis of PBL. 10^6 PBL were stained with appropriately diluted biotinylated monoclonal antibody 40F with 0.1% NaN_3 and fluorescein isothiocyanate (FITC) conjugated avidin. Cells were analysed on EPICS-PROFILE. Transgenic mice were as follows a, NOD; b, C3H; c, $A\beta^k$; d, $A\beta^k$:57Ser; e, $A\alpha^k A\beta^k$; f, $A\alpha^k A\beta^k$:57Ser. B, Immunohistochemical analysis of thymic tissue. Frozen sections (8 μm) of thymus were fixed in acetone-methanol at 4 $^\circ\text{C}$ (10 min) and incubated with 10% mouse serum in PBS at 4 $^\circ\text{C}$ (1 h). After a wash in PBS, the sections were incubated with appropriately diluted biotinylated monoclonal antibody 40F at 4 $^\circ\text{C}$ (1 h) and then with FITC-conjugated streptavidin at 4 $^\circ\text{C}$ (30 min). Each photograph was confirmed to cover both medulla and cortex by comparing with a haematoxylin-eosin stained successive section of each immunostained section. Magnification: NOD, $\times 100$ (a); $A\alpha^k A\beta^k$ transgenic mouse, $\times 100$ and $\times 400$ (b); $A\alpha^k A\beta^k$:57Ser transgenic mouse, $\times 100$ and $\times 400$ (c).

TABLE 1 Incidence of insulinitis

Transgenic mice	Sex	No. of mice	Insulinitis (%)
NOD	F	16	13/16 (81%)
	M	14	12/14 (85%)
NOD- $A\alpha^k$	F	4	3/4 (75%)
	M	4	3/4 (75%)
NOD- $A\beta^k$	F	24	20/24 (83%)
	M	21	18/21 (86%)
NOD- $A\beta^k$:57Ser	F	7	7/7 (100%)
	M	10	10/10 (100%)
NOD- $A\alpha^k A\beta^k$	F	15	5/14 (36%)
	M	21	4/15 (27%)
NOD- $A\alpha^k A\beta^k$:57Ser	F	5	1/5 (20%)
	M	7	1/7 (14%)

Partial pancreatectomy was performed at 20 weeks of age. Dissected tissues were fixed in 10% formalin containing 75 mM phosphate buffer (pH 7.0). After embedding in paraffin blocks, 3- μ m-thick sections were cut and stained with haematoxylin and eosin. More than 20 islets for each pancreas were examined. A pancreas with lymphocytic infiltration around or into at least more than one islet was regarded as displaying insulinitis^{14,15}.

and cortex of thymus (Fig. 1B).

To examine the effect of transgenic I-A expression, partial pancreatectomy was performed at 20 weeks of age, and the presence of insulinitis was analysed histochemically. The incidence of insulinitis in $A\beta^k$ transgenic mice was the same as that of nontransgenic NOD mice (Table 1), in agreement with our previous findings¹⁵. The failure of prevention might be due to the low level of expression of I-A^{d/k} molecules. It is of interest that in $A\alpha^k A\beta^k$ transgenic mice, the incidence of insulinitis decreased to about one-third of that in nontransgenic NOD mice (Table 1). This partial prevention of insulinitis is not directly related to the level of I-A^k expression, because insulinitis could occur in such a transgenic mouse expressing three times as much I-A^k as a C3H mouse (Table 2a). But the number of Langerhans islets with lymphocytic infiltration was small (usually 5–12%), suggesting that diabetes will not occur in these transgenic mice.

Because this partial prevention could be due to the decreased level of endogenous I-A^{NOD} expression by the association of endogenous $A\alpha$ chain with $A\beta^k$ chain, we examined the level of I-A^{NOD} expression. To determine the level of I-A^{NOD} expression in $A\alpha^k A\beta^k$ transgenic mice, we stained the PBL with monoclonal antibody 10.2.16, which can detect I-A^k and I-A^{NOD}, after blocking I-A^k molecules with monoclonal antibody 40F. In $A\alpha^k A\beta^k$ transgenic mice, endogenous I-A^{NOD} molecules were expressed at the same level as in nontransgenic NOD mice (data not shown), suggesting that the prevention of insulinitis is the direct effect of the expression of I-A^k molecules. But, unlike I-E transgenic mice, the prevention of insulinitis was not complete in $A\alpha^k A\beta^k$ transgenic mice. These results indicate that the mechanism of prevention in I-A^k transgenic mice might be different from that in I-E transgenic mice, although the negative selection of autoreactive T cells in thymus has been suggested in I-E transgenic mice^{14,15,20–22}. In any case, our data suggest that the presence of the unique I-A^{NOD} in conjunction with the lack of I-E is indispensable for the development of insulinitis in NOD mice. It is possible that expression of I-A^k molecules might nonspecifically suppress the immunocompetence of the transgenic mice, leading to the prevention of diabetes²³. But we regard this as unlikely because normal immune responses are retained against several antigens so far tested in I-A^k-expressing transgenic mice (data not shown).

To examine the importance of residue 57 of the $A\beta^{\text{NOD}}$ chain, we produced $A\beta^k$:57Ser transgenic mice. To our surprise, insulinitis has occurred without exception. Moreover, the degree

of insulinitis of each mouse was more severe (data not shown) and one male and two females out of 17 mice developed diabetes at the age of 4, 7 or 10 weeks. So far, such early onset of diabetes has not been observed in nontransgenic NOD mice⁸. By contrast, in $A\alpha^k A\beta^k$:57Ser transgenic mice, development of insulinitis was not only prevented as in $A\alpha^k A\beta^k$ transgenic mice, but surprisingly the incidence was lower (Table 1). This was despite the lower level of expression of I-A^k:57Ser molecules as compared with I-A^k expression in $A\alpha^k A\beta^k$ transgenic mice (Table 2b). These results suggest that the single amino-acid substitution from aspartic acid to serine at position 57 of the $A\beta$ chain is not sufficient for development of insulinitis, but that the amino acid at position 57 of the $A\beta$ chain is involved in the interaction between MHC class II molecules and the T-cell receptor of the autoreactive T cells, in the presence or absence of the nominal autoantigen in the thymus or periphery²⁴.

The difference of incidence between $A\alpha^k A\beta^k$:57Ser transgenic mice and $A\beta^k$:57Ser transgenic mice suggests that the $A\alpha$ chain is also involved, probably in the determination of the conformation of I-A molecule. This result is consistent with earlier findings that a specific combination between DQA1 and

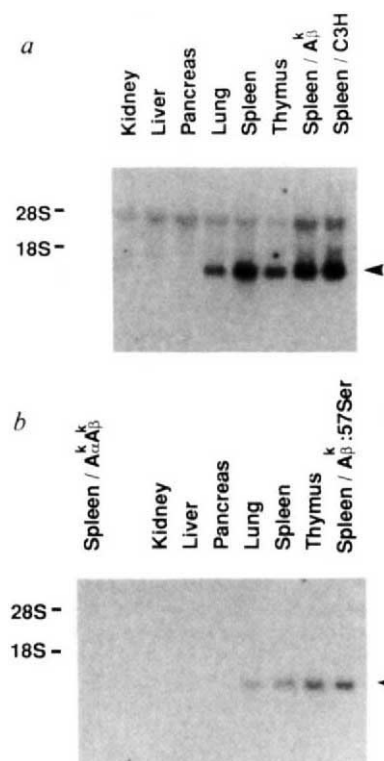


FIG. 2 Northern blot analysis. *a*, Various tissues of an $A\alpha^k A\beta^k$ transgenic mouse and spleen of an $A\beta^k$ transgenic mouse and a control C3H mouse. Total RNA (8 μ g) of each tissue was separated by 1.0% agarose gel electrophoresis, then blotted onto Biodyne membrane (PALL Bio-Support, USA) and hybridized with an oligonucleotide (5'-CAGAAGGGCTGGAACTGGTGC-3'; melting temperature T_m =68 °C) probe, 5' end-labelled with γ [³²P]ATP, in 4 $\times$ SSC, 5% dextran sulphate, 1% SDS at 52 °C (8 h). This oligonucleotide corresponds to the coding sequences of amino acids at positions 9–14 of $A\beta^k$ chain. Blotted membrane was washed at 58 °C in 4 $\times$ SSC, 1% SDS (40 min) and exposed for 72 h. *b*, Tissues of an $A\alpha^k A\beta^k$:57Ser transgenic mouse, and spleen of an $A\beta^k$:57Ser transgenic mouse and of an $A\alpha^k A\beta^k$ transgenic mouse. Oligonucleotide probe: 5'-GTACTCGGCGGATGGCCGCC-3' (T_m =78 °C), corresponding to the coding sequences of the amino acids at positions 54–60 of the $A\beta^k$:57Ser chain, had been used also for site-directed mutagenesis of position 57 (underlined sequence) from GAC to TCC. Blotted membrane was washed at 70 °C in 4 $\times$ SSC, 1% SDS (40 min). Other procedures were as described in *a*. There is no detectable signal on the lane of spleen of the $A\alpha^k A\beta^k$ transgenic mouse. Positions of 28S and 18S ribosomal RNA are shown on the left.

TABLE 2 Insulinitis and the level of transgenic products

	Mouse	Sex	Insulinitis	Level of I-A ^k (%)	I-A ^k positive PBL (%)
(a)	1	M	+	43	23
	2	F	+	108	75
	3	F	+	105	23
	4	M	+	124	45
	5	F	+	147	38
	6	F	+	187	48
	7	F	+	193	51
	8	M	+	293	57
	9	M	+	347	52
	10	F	—	75	77
	11	F	—	89	31
	12	F	—	98	33
	13	M	—	102	57
	14	M	—	136	48
	15	F	—	183	30
	16	F	—	185	52
	17	F	—	195	61
	18	F	—	205	44
	19	M	—	214	58
	20	F	—	224	45
	21	M	—	235	80
	22	M	—	243	67
	23	M	—	253	81
	24	M	—	307	79
	25	M	—	340	81
	26	M	—	342	59
	27	M	—	370	63
	28	M	—	425	28
	29	F	—	872	73
(b)	1	M	+	79	50
	2	F	+	127	17
	3	M	—	48	65
	4	F	—	68	30
	5	M	—	81	34
	6	M	—	95	44
	7	M	—	96	39
	8	M	—	100	17
	9	F	—	113	34
	10	F	—	120	21
	11	F	—	123	49
	12	M	—	134	48

Immunofluorescent analysis was performed for PBL of A α^k A β^k transgenic mice and A α^k A β^k :57Ser transgenic mice. Level of expression is represented by the mean fluorescent intensity of positive cells. Level of expression and the percentage of positive cells are presented as the relative figures as compared with those of a C3H mouse which has k-haplotype. For analysis, cells were treated as described in Fig. 1a. a, A α^k A β^k transgenic mice. b, A α^k A β^k :57Ser transgenic mice.

DQB1 genes contributes to susceptibility to human insulin-dependent diabetes mellitus²⁵.

Recent evidence suggests that the lack of I-E is one of the key factors for the development of insulinitis in NOD mice^{14,15,22}. There has, however, been no direct evidence for the contribution of the unique I-A^{NOD} to the development of insulinitis. Here we have clearly demonstrated that the unique I-A^{NOD} plays an important part in the development of insulinitis, and that residue 57 is involved in the conformational change of I-A molecule in such a way as to alter the interaction with the T-cell receptor, although the single amino-acid substitution of aspartic acid with serine at position 57 of the A β^k chain is not sufficient for the susceptibility to autoimmune insulinitis. □

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Prevention of diabetes in non-obese diabetic I-A^k transgenic mice

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THE non-obese diabetic (NOD) mouse develops insulin-dependent diabetes mellitus (IDDM) with mononuclear cell infiltration of the islets of Langerhans and selective destruction of the insulin-producing β -cells, as in humans¹. Most infiltrating cells are T lymphocytes, and most of these carry the CD4 antigen². Adoptive transfer of T cells from diabetic NOD mice into irradiated NOD or athymic nude NOD mice induces diabetes³. Susceptibility to IDDM in NOD mice is polygenic⁴, with one gene linked to the major histocompatibility complex class II locus⁵, which in NOD mice expresses a unique I-A molecule but no I-E. Speculation exists as to the role of the I-A molecule in the diabetes susceptibility of NOD mice, especially regarding the significance of specific unique residues^{6,7}. To examine the role of the NOD I-A molecule in IDDM pathogenesis, we made NOD/Lt mice transgenic for I-A^k by microinjecting I-A^k α - and β -genes into fertilized NOD/Lt eggs. Insulinitis was markedly reduced and diabetes prevented in NOD/Lt mice expressing I-A^k.

To determine whether the unique I-A molecule of the diabetes-prone NOD/Lt mouse contributed to the disease process, we introduced the I-A^k genes into NOD/Lt mice using transgenic technology. I-A^k transgenic NOD/Lt mice were produced by microinjecting a 9.3 kilobase (kb) *HindIII* fragment containing the entire I-A^k α -gene⁸ (including 3 kb of 5' flanking sequence) and a 14 kb *XmnI* fragment containing the entire I-A^k β -gene⁹ (including 3 kb of 5' flanking sequence and 2.75 kb of 5' pBR322 vector sequence). Mice were screened for the transgene by Southern blot analysis of tail DNA using ³²P-labelled I-A^k α - and β -specific complementary DNA probes. Five transgenic mice were obtained that had integrated both α - and β -genes. Of these, two were sterile males, one was a non-expressing male and two were females that expressed I-A^k. These founder females were mated to nontransgenic NOD/Lt mice and the offspring screened by dot-blot analyses using the 1.4-kb *PstI-SalI* fragment from plasmid pAT153.

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Transgenic offspring of one founder female expressed $I-A^k$ at levels comparable to that of CBA ($H-2^k$) mice, and this lineage was established for experimentation (data not shown).

To characterize expression of the transgene product, flow cytometric analyses of spleen, lymph node, bone marrow and thymus cells were performed using monoclonal antibodies specific for $I-A^k\alpha$ and β . Both the α - and β -chains of the $I-A^k$ heterodimer were expressed on spleen, lymph node and thymus cells but not on bone marrow cells (Fig. 1). It is unlikely that mixed haplotype pairing occurred between NOD/Lt $I-A$ and $I-A^k$ molecules in view of the restricted assembly of class II $I-A^k$ molecules *in vivo*¹⁰. Spleen and lymph node cells expressing $I-A^k$ carried the B220 antigen, but not Thy-1.2 (data not shown). Immunofluorescent staining of thymus cryostat sections with anti- $I-A^k$ antibodies showed an expression pattern of the transgene similar to that of class II major histocompatibility complex (MHC) genes, although staining of thymus cortex was weaker in NOD/Lt- $I-A^k$ transgenic mice (Fig. 2). Nontransgenic NOD/Lt mice, irradiated and reconstituted with transgenic bone marrow, expressed $I-A^k$ only in the thymus medulla by 3 weeks post-reconstitution in cells having a dendritic appearance (Fig. 2d). Hence, despite the lack of $I-A^k$ cells in bone marrow (Fig. 1), marrow-derived cells do express the transgene in the thymus medulla.

$I-A^k$ was also expressed in immunogenic form in the skin of NOD/Lt- $I-A^k$ transgenic mice. This was shown by the rejection of 11 out of 12 sex-matched grafts of transgenic tail skin by 10-week-old NOD/Lt mice within 13 days. Furthermore, the transgenic $I-A^k$ was functional in a restriction assay using two different hen egg lysozyme (HEL)-specific, $I-A^k$ -restricted T-cell hybridomas. The transgenically expressed $I-A^k$ molecules could associate intracellularly with processed foreign antigen and restrict the presentation of the synthetic peptide HEL 46-61 (Fig. 3).

None of the transgenic mice developed diabetes: of 26 female mice assessed at 150 days, and 10 at 250 days, none were hyper-

glycaemic. In marked contrast, female NOD/Lt mice developed diabetes at an incidence of 46% at 150 days and 93% at 250 days. The complete lack of diabetes in the transgenic mice was not a feature of breeding conditions, as the incidence of diabetes in 27 female nontransgenic littermates at 135 days was 22%, not significantly different from the incidence in sex and age-matched NOD/Lt mice. To assess the effect of the expression of the $I-A^k$ transgene on insulinitis, we biopsied the body and tail of the pancreas of 23 transgenic and 20 nontransgenic littermates, aged between 112–300 days. The pancreata were examined for the presence of insulitis and were scored for severity of infiltration as described¹¹. Transgenic mice had a significantly reduced 'insulitis score' compared with littermate controls (Fig. 4). In contrast to nontransgenic NOD/Lt littermates, most of the islets in the transgenic mice biopsies were completely free of insulitis. The presence of some insulitis, however, in isolated islets of some transgenic mice is difficult to explain. Although in a previous experiment back-crossing the $I-Ea^d$ transgene into NOD mice completely prevented insulitis, back-crossing chromosome 17 (which has the $I-A^b$ locus) prevented insulitis in only 95% of cases¹², suggesting that the diabetogenic effect of the NOD $I-A$ may be dominant but have low penetrance¹³.

The protective effect of $I-A^k$ is unlikely to be a nonspecific effect of transgene incorporation, as diabetes was not prevented when only the $I-A\beta^k$ gene was introduced into NOD mice¹⁴. Nor could it be due to a defect in immune competence, because the protected transgenic mice rejected skin allografts (four out of four transgenic rejected CBA skin within 12 days, compared with five out of six littermates, the sixth one rejecting by day 14).

Recent evidence has implicated T cells with particular $V\beta$ region gene usage in the pathogenesis of IDDM. Diabetes was transferred to sublethally irradiated young NOD mice by T-cell clones bearing receptors encoded by a $V\beta 5$ gene segment¹⁵. Cyclophosphamide-induced diabetes was prevented in NOD mice by a monoclonal antibody directed to T cells bearing receptors encoded by a $V\beta 8$ gene segment¹⁶. To determine

FIG. 1 Expression of the transgene products $I-A^k\alpha$ and $I-A^k\beta$ detected by flow cytometric analyses of spleen, lymph node, bone marrow and thymus cells. *a, e, i, m*, Spleen, lymph node, bone marrow and thymus cells respectively from NOD/Lt- $I-A^k$ transgenics (solid line) and nontransgenic littermates (dotted line) stained with fluorescein-conjugated monoclonal 39J (anti- $I-A^k\alpha$)²⁴. *c, g, k, o*, Stained with monoclonal 40F (anti- $I-A^k\beta$)²⁴. *b, f, j, n*, Cells from tissues of CBA ($I-A^k$) mice stained with anti- $I-A^k\alpha$. *d, h, l, p*, Stained with anti- $I-A^k\beta$.

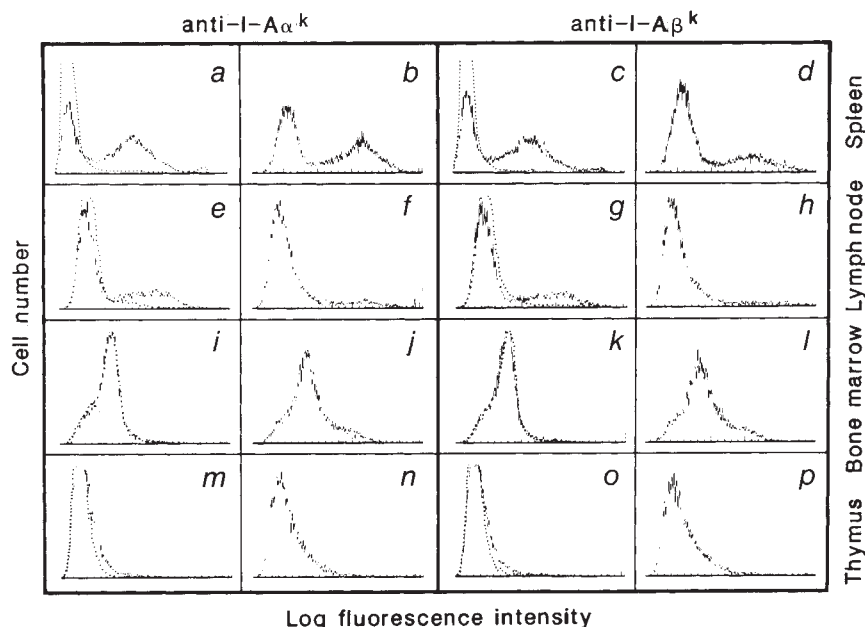
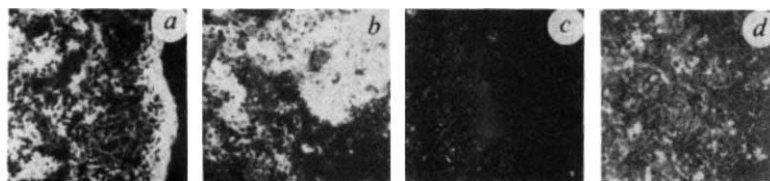


FIG. 2 Expression of the transgene product $I-A^k\alpha$ in the thymus of NOD/Lt- $I-A^k$ transgenic mice. $I-A^k$ expression in CBA thymus (*a*), NOD/Lt- $I-A^k$ transgenic thymus (*b*), NOD/Lt nontransgenic thymus (*c*) and bone marrow chimaeric thymus (*d*) 7 weeks post-reconstitution. Magnification, $\times 150$. METHODS. Chimaeric mice were made by irradiating 6-week-old female nontransgenic NOD/Lt mice with 950 rads and reconstituting with 5×10^6 NOD/Lt- $I-A^k$ transgenic bone marrow cells. Cryostat sections 6 μ m were stained with fluorescein-conjugated anti- $I-A^k\alpha$.



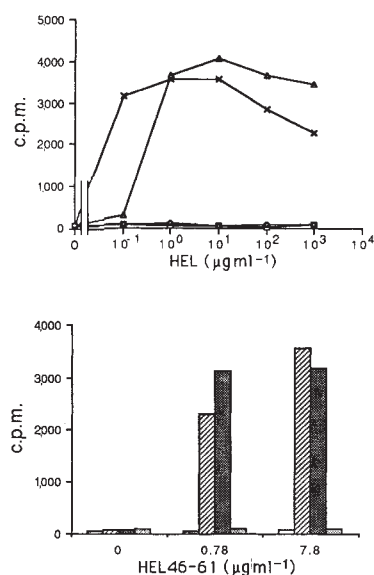


FIG. 3 Ability of transgenic I-A^k molecules to restrict the presentation of native hen egg lysozyme (HEL) and the synthetic peptide HEL 46-61, to two HEL 46-61 specific, I-A^k-restricted T-cell hybridomas in a manner essentially indistinguishable from wild-type I-A^k. *a*, Response of 3A9 hybridoma to HEL. Transgenic NOD/Lt-I-A^k, Δ ; NOD/Lt littermate, $\square$; B10.A(4R), $\times$; C57BL/6, $\diamond$. *b*, Response of 3A9 hybridoma to HEL 46-61. Transgenic NOD/Lt-I-A^k, $\boxtimes$; NOD/Lt littermate, $\square$; B10.A(4R), $\blacksquare$; C57BL/6, $\blacksquare$. The response by hybridoma A2.2B2 gave similar results. For clarity, only results obtained using one mouse of each group are plotted; comparable results were obtained with three other mice from each group (except for the C57BL/6 group in which only two mice were tested).

METHODS. Supernatants from 24-h co-cultures containing 10⁵ of the HEL 46-61-specific, I-A^k-restricted T-cell hybridomas A2.2B2 or 3A9 (ref. 25) and 5 $\times$ 10⁵ splenic antigen-presenting cells in the presence or absence of native HEL or HEL 46-61 (final volume, 200 μ l) were freeze-thawed and assayed for interleukin-2 (IL-2) content by proliferation of the IL-2-dependent cell line, CTLL. For the proliferative assay, 5 $\times$ 10³ CTLL cells were cultured in 25% v/v test supernatant (final volume, 200 μ l) and pulsed with 0.1 μ Ci [¹²⁵I] 5-iodo-2-deoxyuridine at 18 h. Cells were collected on a cell harvester 6 h later. Assays were carried out in triplicate and background counts were between 50 and 100 c.p.m. Titration of test supernatants routinely gave similar results to those obtained with 25% v/v.

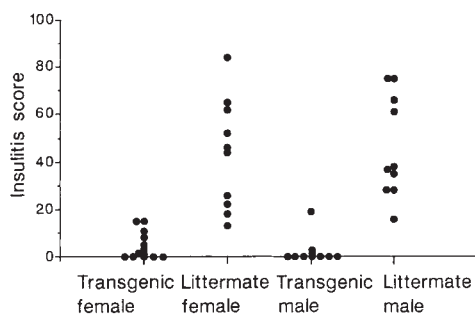


FIG. 4 Incidence of insulinitis in 23 NOD/Lt-I-A^k transgenic mice and 20 nontransgenic littermate control mice aged between 112 and 300 days. The pancreata were examined for the presence of insulinitis which was scored according to a graded system of severity of infiltration¹¹.

METHODS. The pancreas was cut at three levels and the islets were blindly scored on a 0-4 grading scale. Where sufficient numbers of islets were seen (>12), this was converted to a percentage score. Islets free of any peri-islet mononuclear cells were scored as 0; a focal peri-islet circumference as 1; a more extensive peri-islet aggregate as 2; intra-islet infiltration with preservation of β -cells and retention of islet shape as 3; and extensive intra-islet infiltration with obvious β -cell damage and gross distortion of islet morphology as 4. A score of 4 also included a residual end-stage islet with complete loss of β -cells. If all islets were destroyed, the score would be 100%.

whether there was a change in the overall spleen cell numbers of T-cell clones using V β 5, V β 6, V β 8 and V β 11 region genes, we analysed the cells by flow cytometry with appropriate monoclonal antibodies. No significant differences were found between transgenic and control mice (data not shown).

I-A^{B^{NOD}} is distinct from I-A^k as well as from the I-A of most other strains at several amino-acid residues including residue 56 and 57 (ref. 6). The protective effect of I-A^k gene expression in NOD/Lt mice is probably due to some distinct features of its overall structure. Further studies are required to locate precisely the significant residues. Whether a single residue or a specific conformation of residues is involved, the defect that leads to autoimmune insulinitis may be the failure to present a particular peptide which would readily be bound by the I-A^k molecule. Presentation of such a peptide intrathymically may be essential to allow either positive selection of T cells^{17,18}, which would exert a protective effect, or negative selection of T-cells^{19,20} reactive against the putative islet cell antigen. In the former case, positive selection of T cells with suppressor activities would prevent responses to the islet antigen. Defects in suppression have indeed been proposed for the pathogenesis of diabetes in both humans²¹ and NOD mice^{11,22}. Alternatively, if negative selection is not operating efficiently in NOD mice, because of the low affinity of binding of a particular peptide to the NOD class II molecules, T cells with the potential to respond to the putative antigen and initiate disease would peripheralize. They might be activated if, following a trigger such as a viral infection of the β -cells, the antigen is upregulated to a level at which it can now be effectively presented in association with NOD class II molecules²³. If this is the mechanism of protection afforded by I-A^k, the effect may be transferred by simply injecting transgenic bone marrow into neonatal NOD/Lt mice to provide a source of transgenic dendritic cells.

By using transgenic technology, therefore, we have demonstrated that the expression of I-A^k in NOD/Lt mice completely prevents diabetes and markedly reduces insulinitis. Our results may also be relevant to human diabetes, which has also been linked to particular susceptibility haplotypes. Thus the existence of particular class II molecules may predispose to IDDM by influencing the adequate presentation of putative disease-associated peptides and thereby the selection of the T-cell repertoire. This model remains valuable for defining more precisely the mode of action of I-A in the disease process. $\square$

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Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A β -chain or normal I-E α -chain

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INSULIN-dependent diabetes mellitus (IDDM) is a disease with an autoimmune aetiology. The inbred non-obese diabetic (NOD) mouse strain provides a good animal model of the human disease¹ and genetic analysis suggests that, as in man, at least one of the several genes controlling the development of IDDM is linked to the major histocompatibility complex^{2,3}. The NOD mouse does not express I-E² owing to a deletion in the promoter region of the I-E α -chain gene, and the sequence of NOD I-A β -chain in the first external domain is unique with His 56 and Ser 57 (ref. 4) replacing Pro and Asp, respectively, at these positions. There has been considerable interest in the role amino acid 57 might have in conferring susceptibility to autoimmune diseases, including IDDM. The presence of a charged residue (such as Asp) at this position might affect the conformation of the peptide binding groove⁵. But it could be assumed that Pro 56 gives rise to a different conformation of I-A β -chain than does His 56. We therefore constructed transgenic NOD mice in which the transgene encoded a modified A β ^{NOD} with Pro 56, and studied its effect on the development of IDDM in this mouse strain. Previous studies have suggested that NOD mice expressing I-E as a result of the introduction of an I-E α -chain (E α) transgene are protected from the development of insulinitis and hence IDDM^{6,7}. To explore further the protective effect of this molecule we constructed a second class of transgenic NOD mouse carrying an E α ^d transgene. Both transgenes protected the mice from IDDM, but this was not associated with a complete deletion of any T cells expressing commonly used T-cell receptor V β genes.

To test the hypothesis that residue 56 in the NOD A β molecule is important in the development of insulinitis and disease, we generated NOD mice carrying a mutated A β ^{NOD} gene (see Fig. 1a). Two point mutations were introduced into NOD A β to generate the sequence encoding a Pro 56 instead of His. This destroys a restriction site for *DdeI* at this position in the A β ^{NOD} gene. Several founder transgenic NOD mice were generated and the progeny of one of these, NOD-A-PRO-3, analysed in more detail. The transgene is clearly expressed in these mice as amplification using the polymerase chain reaction (PCR) of complementary DNA generated from spleen RNA of NOD-A-PRO-3 mice, gave a product which could only partially be digested by *DdeI* (Fig. 1c) and which was detected by an oligonucleotide probe specific for sequences encoded by the mutated region of the transgene (Fig. 1d). The *DdeI*-sensitive component present in the NOD-A-PRO-3 progeny derives from the endogenous A β ^{NOD} gene. Histological analysis of NOD-A-PRO-3 transgenic mice showed a clear effect of the transgene on pancreatic infiltration. There was no evidence of intra-islet

TABLE 1 Disease incidence and immunohistochemical analysis of female NOD and NOD transgenic mice

Mouse line	Islets showing maximal intra-islet infiltration (%)	Islets showing peri-islet infiltration (%)	Incidence of diabetes
NOD	59	29	20/31
NOD-A-PRO-3	0	47	0/8
NOD-E-3	0	16	0/36
NOD-E-9	0	46	0/11

Immunohistochemical analysis was performed of pancreatic cryostat sections, double stained with guinea-pig anti-insulin antibodies and OX-6 (ref. 15), a mouse monoclonal antibody that recognises NOD class II MHC¹⁶. No severe intra-islet infiltration was seen in any of the transgenic animals. The peri-islet infiltration seen in transgenic mice never exceeded 20 cells in any given islet area analysed. We examined 144 islets from NOD mice, 299 from NOD-A-PRO-3, 85 from NOD-E-3 and 92 from NOD-E-9 transgenic mice. Diabetes was diagnosed on the basis of presence in the urine of glucose (>111 mM⁻¹) determined by Diastix (Ames) on two or more subsequent occasions, 1 week apart. The incidence of diabetes in NOD, NOD-E-3 and NOD-E-9 female mice is the cumulative incidence at one year. That of the NOD-A-PRO-3 was from eight females aged 20–40 weeks.

TABLE 2 Mitogen- and antigen-stimulated proliferative responses of NOD-A-PRO-3 transgenic mice

Mouse number	Transgene expression	Mitogen			Antigen		
		medium	LPS	Con A	self	B10	CBA
C1	–	6*	29	166	7	29	27
C2	–	9	22	125	7	35	35
C3	+	3	23	160	3	9	10
C4	+	6	24	183	2	11	10
C5	–	3	21	171	1	8	11
C6	–	6	26	118	5	12	17
C7	+	2	13	126	3	10	9
C8	+	2	19	142	2	11	17

Red blood cell-free (rbcf) responder spleen cells at 2×10^5 per well were stimulated with 5 μ g per well lipopolysaccharide S (LPS), or 0.2 μ g per well concanavalin A (Con A), or cocultured with 5×10^5 cells per well of 2,000R irradiated rbcf spleen cells from self, C57BL/10 (B10) or CBA, in 96-well flat-bottomed microtitre plates, containing a final volume of 200 μ l per well of bicarbonate-buffered RPMI 1640 medium supplemented with 10% FCS, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 2 mM L-glutamine. Plates were incubated in a 5% CO₂ humidified air incubator at 37 °C. Each well of the mitogen-stimulated plates was pulsed with 1 μ Ci [³H]thymidine (after 63 h) and collected 7 h later using the Betaplate system. Antigen-stimulated plates were similarly treated except that the [³H]thymidine was added after 87 h.

* Counts expressed as c.p.m. $\times 10^{-3}$.

infiltration by mononuclear cells or of β -cell destruction in the pancreas of NOD-A-PRO-3 mice, whereas the pancreas of transgene-negative littermate controls showed massive intra-islet infiltration by T cells and macrophages (data not shown) and β -cell destruction (Table 1). Accumulation of mononuclear cells in a peri-islet location is common in the pancreas of NOD mice from 5 weeks of age, and occurs irrespectively of the subsequent development of diabetes. NOD-A-PRO-3 transgenic mice showed a marked reduction in the numbers of islets with peri-islet infiltration, as well as a reduction in the numbers of cells present in such infiltrates. None of these developed diabetes by the age of 5 months. To examine whether the introduction of this transgene had affected immune responses in general, spleen cells from NOD-A-PRO-3 mice and their transgene-negative littermates were tested for their ability to respond to mitogens and alloantigens (Table 2). No significant differences in their immune responses could be detected. The introduction of any A β transgene into NOD mice is not sufficient to protect from

insulinitis, as indicated by the finding that introduction of an $A\beta^k$ transgene into NOD mice does not prevent intra-islet infiltration⁷.

To study the protective mechanisms of the I-E molecule, we generated transgenic mice carrying the Ea^d transgene (Fig. 1b) which restores expression of I-E. Progeny of two founders, NOD-E-3 and NOD-E-9 have been analysed. As in the NOD-A-PRO-3 transgenic animals, none of the I-E-expressing mice developed IDDM and only minimal peri-islet infiltration was observed (Table 1). Although female NOD mice have a higher incidence of IDDM than male NOD mice⁸, disease can be induced in males following administration of high doses of cyclophosphamide or transfer of diabetic spleen cells⁹. Treatment of male NOD mice with cyclophosphamide (200 mg kg⁻¹ administered intraperitoneally on days 0 and 14) induced IDDM in 4 out of 10 animals, but none of the 11 NOD-E-3 transgenic mice treated with cyclophosphamide developed IDDM. IDDM can be transferred to non-diabetic recipients by diabetic spleen cells¹⁰. When diabetic spleen cells from NOD mice were transferred into irradiated NOD-E-3 mice, the recipients developed a graft versus host disease and severe intra-islet infiltration with evidence of β -cell destruction. This suggests that the expression of I-E on the transgenic tissue does not affect the

expression of the autoantigen.

Another factor in the pathogenesis of IDDM could be the overexpression of major histocompatibility complex (MHC) class I molecules associated with infiltrated islets. In the pre-diabetic NOD mouse pancreas, an increased expression of class I MHC antigen is seen over the whole islet area and extends into the exocrine tissue, concomitant with the intra-islet infiltration by mononuclear cells (data not shown). This pattern of class I expression is not seen in the pancreas of normal mouse strains or our transgenic animals (NOD-A-PRO-3, NOD-E-3, NOD-E-9). The class I expression in these animals is restricted to tissue macrophages and to blood vessels (data not shown). NOD mice develop not only a mononuclear cell infiltration in the pancreatic islets, but also an infiltration in the salivary gland resembling that seen in human Sjogren's syndrome. In the submandibular salivary glands of NOD mice, expression of class II MHC is detected on the ductal epithelium adjacent to areas of infiltration. A similar pattern of class II MHC expression was observed in the glands of the transgenic mice (data not shown). Moreover, severe infiltration of these glands was detected in both NOD-E and NOD-A-PRO-3 transgenic mice, in contrast to the findings in the pancreas indicating that the transgenes had no effect on salivary infiltration. Hence, the

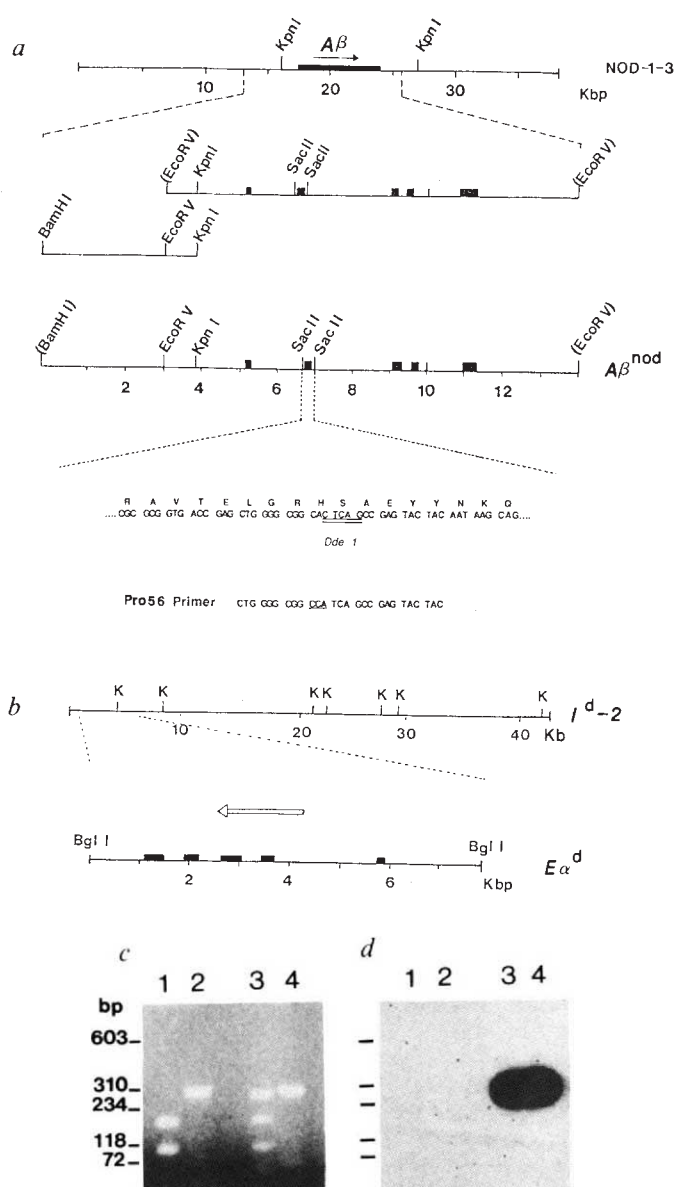


FIG. 1 a, Restriction map of cos NOD-1-3 containing the entire NOD $A\beta$ gene isolated from a NOD liver genomic library. The $A\beta$ NOD-PRO transgene was subcloned in three steps. First, an 11.0-kb *EcoRV* fragment containing the structural gene for $A\beta^{\text{NOD}}$ was subcloned using *Clal* linkers into plasmid pSK⁺ deleted for the *SacII* site. For *in vitro* mutagenesis, the 330-base pair (bp) *SacII* fragment containing exon II was subcloned into pSK⁺ and used to change the residue 56 from His to Pro. Second, to ensure sufficient 5' flanking sequences for tissue-specific expression of the final transgene, a 4.2-kb 5' *BamHI*-*KpnI* flanking fragment of the $A\beta$ NOD-PRO gene was inserted into the *SacI*-*KpnI* site of the pTCF plasmid¹⁷. Third, the 10.4 kb *KpnI*-*Clal* fragment of the $A\beta$ NOD-PRO gene was inserted into the *KpnI*-*Clal* site of the 5' flanking subclone. The reconstituted $A\beta$ NOD-PRO transgene devoid of plasmid sequences was injected into fertilised mouse embryos. b, Restriction map of cos I^d-2, isolated from a genomic cosmid library together with cos I^d-1 (ref. 18). A 7.5-kb *BglI* fragment containing the Ea^d gene and 2 kb 5' and ~2 kb 3' flanking sequences, was subcloned using *Clal* linkers into pSK⁺ and injected into fertilized mouse embryos after removal of bacterial sequences. K, *KpnI* sites. c, Ethidium bromide-stained agarose gel of nucleotides 97-385 of $A\beta$ messenger RNA (numbering from ref. 4) from total spleen RNA. Lanes 1, 2, NOD spleen cDNA with and without digestion with *DdeI*; lanes 3, 4, NOD-A-PRO-3 spleen cDNA with and without *DdeI* digestion. Size markers in base pairs are from ϕ X174 DNA digested with *HaeIII*. d, Autoradiogram of the blot hybridized with the labelled $A\beta$ -PRO specific primer.

METHODS. a, b, The cos NOD-1-3 clone was isolated from a genomic cosmid constructed as described¹⁷ from NOD/CRC liver DNA by screening with a 1.5-kb *BamHI*-*HindIII* fragment from the $A\beta^b$ gene containing exons III and IV¹⁸. For *in vitro* mutagenesis, a single-stranded template of the $A\beta$ exon II subclone was isolated following superinfection with the VCM13 helper phage (Stratagene). The mutagenesis was carried out using PRO primer as specified above and a mutagenesis system (Amersham) as recommended by the manufacturer. c, d, Total RNA was extracted from NOD and NOD-A-PRO-3 as described²⁰. Complementary DNA was synthesized from 10 μ g RNA (Amersham cDNA synthesis system) at 42 °C (90 min) in a volume of 20 μ l; cDNA synthesis was terminated by adding 180 μ l PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin). PCR was performed using as primers 5-GCAGCCAGGGACTGAGGGC (sense primer; corresponding to the last 20 nucleotides of exon 1) and GGGAACCTCCGTCTCC (anti-sense primer; complementary to nucleotides 366-385 of $A\beta$, ref. 4) in a Hybaid thermal reactor, by heating to 95 °C (3 min) followed by 36 cycles of 90 °C (1 min), 55 °C (2 min) and 72 °C (3 min). The amplified DNA was phenol-chloroform extracted and ethanol-precipitated in the presence of glycogen. After 2 h at -20 °C, the DNA was dissolved in 100 μ l H₂O; 10 μ l was digested with 5 U *DdeI*. The digested sample and 10 μ l undigested PCR product were electrophoresed in a 1.2% agarose gel containing ethidium bromide, and photographed. The gel was then soaked in denaturing solution (0.4 M NaOH) and transferred to a Zetabind membrane in 0.4 M NaOH overnight. The membrane was hybridized with end-labelled primer GGCTGATGGCCG at 42 °C (150 min) followed by washing with 2 $\times$ SSC, 0.1% SDS and autoradiographed at -70 °C (90 min).

TABLE 3 T-cell receptor $V\beta$ use of peripheral T cells in NOD and NOD transgenic mice

	CD4			
	$V\beta 5$	$V\beta 6$	$V\beta 8$	$V\beta 11$
NOD	1.2 ± 0.3	1.5 ± 1.2	29.6 ± 0.5	4.7 ± 1.1
NOD-E-3	0.7 ± 0.3	0.9 ± 0.8	28.2 ± 1.6	4.2 ± 1.9
NOD-A-PRO-3	0.9 ± 0.5	1.2 ± 1.6	29.6 ± 0.9	4.3 ± 1.4
	CD8			
	$V\beta 5$	$V\beta 6$	$V\beta 8$	$V\beta 11$
NOD	6.9 ± 1.5	1.9 ± 0.9	29.2 ± 3.1	4.7 ± 1.6
NOD-E-3	2.1 ± 1.3	2.3 ± 0.9	33.8 ± 5.5	7.1 ± 2.6
NOD-A-PRO-3	5.9 ± 1.7	2.0	27.4 ± 3.3	5.0 ± 1.6

Cervical and axillary lymph nodes of 3–6-month-old mice were made into cell suspensions at $1-2 \times 10^7$ cells per ml in PBS containing 2% newborn calf serum and 0.1% sodium azide. To 50- μ l aliquots in tubes were added each of the $V\beta$ -specific monoclonal antibodies (culture supernatants). After a 20-min incubation at 4 °C, the cells were washed once and then anti-mouse immunoglobulin coupled to fluorescein (FITC) added, to give a final concentration of 1:25. After a further 4 °C incubation for 20 min and one wash, the cells were blocked by addition of normal mouse immunoglobulin for 10 min. CD4 antibodies (BD L3T4) coupled to phycoerythrin and CD8 antibodies (KT 15) coupled to biotin were then added to each tube and incubated for 20 min. After washing, streptavidin allophycocyanin was added and incubated for 10 min, followed by a final wash before analysis on a FACStar PLUS. Appropriate gates for CD4 and CD8 were set and 10,000 or 30,000 cells from each sample were counted. The figures given represent the average percentage of cells positive with each $V\beta$ -specific monoclonal antibody, within each of the CD4 and CD8 subclasses respectively, from three to five measurements made on cells from individual mice. The $V\beta 6$ (44-22-1) and $V\beta 8$ (F23.1) monoclonal antibodies were kindly donated by Drs H. Hengartner and P. Marrack respectively. $V\beta 11$ (KT11) and CD8 (KT15) were provided by Dr K. Tomonari.

development of IDDM and salivary infiltration are two separable events in NOD mice.

Genetic data suggests that development of IDDM in NOD mice is controlled by three recessive genes, one of which (*Idd-1*) is linked to the MHC. Although the severe pancreatic islet infiltration seems to be influenced by an MHC gene, the occurrence of minimal pancreatic infiltration is controlled by a gene probably not linked to the MHC³. Our studies in transgenic NOD mice support these findings, because minimal infiltration is still seen around some islets of NOD mice expressing each of the transgenes (Table 1). Introduction of these two transgenes (modified $A\beta^{\text{NOD}}$ or Ea^d) has therefore prevented islet invasion and β -cell destruction in these animals. But the mechanism underlying the protective effect remains unclear.

Expression of I-E modifies the peripheral T-cell repertoire by deletion of T cells expressing certain T-cell receptor $V\beta$ genes^{11,12}. The inability to induce disease in NOD-E-3 transgenics with high doses of cyclophosphamide is consistent with such a deletion mechanism. But from fluorescence-activated cell sorting (FACS) analysis of $V\beta$ use in peripheral T cells, no clear candidate for $V\beta$ deletion is apparent, using the currently available $V\beta$ -specific monoclonal antibodies (Table 3). The NOD-PRO-3 transgenic mice showed no alteration in $V\beta 5$, $V\beta 6$, $V\beta 8$ or $V\beta 11$ use; the quantitative reduction of $V\beta 5$ -positive T cells in NOD-E-3 mice might involve a particular $V\beta 5$ subclass of T cells which could be a candidate¹³. Other data are consistent with a heterogeneous use of $V\beta$ genes in T cells capable of destroying pancreatic β -cells¹⁴ (K. Haskins, personal communication). Furthermore, although transgenic NOD mice showing normal or abnormal tissue distribution of I-E expression deplete $V\beta 5$ -bearing T cells, protection from insulinitis is only observed with wild-type tissue distribution of I-E expression (J. Bohme and D. Mathis, personal communication). Taken together, this suggests that the protective effects of these transgenes may be more complex. It is possible that T-cell receptors using other $V\beta$ and/or $V\alpha$ genes are deleted or that the introduction of new restriction elements has subtly altered the pattern of T-cell responses. For NOD-A-PRO-3 mice, it is also possible that the transgene affects the presentation of the autoantigen. □

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Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A

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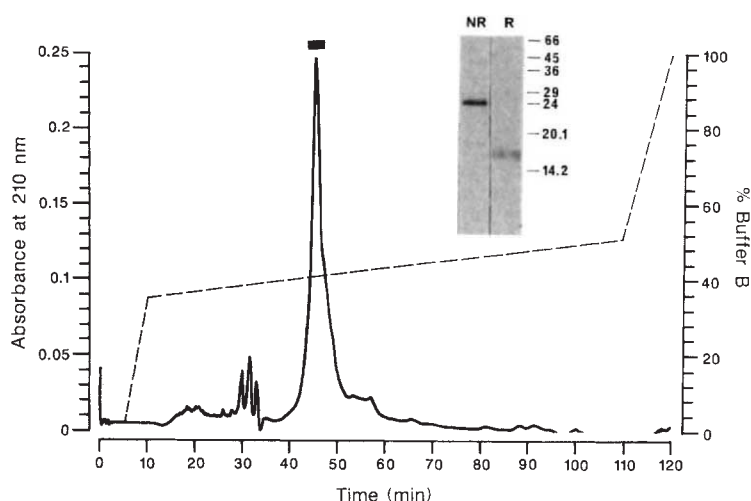
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THE first inductive interaction in amphibian development is mesoderm induction, when a signal from the vegetal hemisphere of the blastula induces mesoderm from overlying equatorial cells^{1,2}. Recently, several 'mesoderm-inducing factors' (MIFs) have been discovered. These cause isolated *Xenopus* animal caps to form mesodermal cell types such as muscle, instead of their normal fate of epidermis. The MIFs fall into two classes. One comprises members of the fibroblast growth factor (FGF) family^{3–5}, and the other members of the transforming growth factor type β (TGF- β) family^{6–7}. Of the latter group, the most potent is XTC-MIF, a protein produced by *Xenopus* XTC cells^{8–10}. Here we show that XTC-MIF is the homologue of mammalian activin A. Activins modulate the release of follicle-stimulating hormone from cultured anterior pituitary cells^{11,12} and cause the differentiation of two erythroleukaemia cell lines^{13,14}. Our results indicate that these molecules may also act in early development during formation of the mesoderm.

XTC-MIF was purified from serum-free XTC-conditioned medium using a modification of our previous protocol⁹. The purified protein eluted as a single peak at about 32% acetonitrile after reverse-phase HPLC (Fig. 1). It migrated as a single band on polyacrylamide gel electrophoresis under both non-reducing and reducing conditions, with relative molecular masses (M_r) of ~24,000 and ~16,000 respectively (Fig. 1 inset). XTC-MIF was quantitated by amino acid analysis and found to be capable of inducing muscle-specific actin gene expression in *Xenopus* animal caps at concentrations of 0.2 ng ml⁻¹ and above (Fig. 2). By contrast, TGF- $\beta 2$ induces muscle-specific actin only at concentrations greater than 3–12 ng ml⁻¹ (ref. 6). N-terminal amino-acid sequencing of XTC-MIF resulted in a single sequence with identity to activin A but not to activin B, the inhibin- α chain, TGF- β s 1–5, Vg1 or decapentaplegic (DPP) (Fig. 3).

This result suggested that XTC-MIF is the *Xenopus*

FIG. 1 Final step in the purification of XTC-MIF. Serum-free XTC-conditioned medium (165 litres) was purified as previously⁹, with modifications. Batches of conditioned medium were concentrated by ultrafiltration and stored at -70°C . The pooled batches were heated to 100°C , cooled, adjusted to 1 M NaCl, and loaded on to a 5×30 cm column of Phenyl-Sepharose CL-4B. After washing, the column was eluted with a gradient of ethylene glycol. On assay⁹, activity eluted at $\sim 40\%$ ethylene glycol. Active fractions were then applied to a 2.6×60 cm column of DEAE-Sepharose CL-4B. After washing, the column was eluted with a gradient of NaCl; mesoderm-inducing activity eluted at ~ 0.6 M NaCl. Active fractions from this step were then adjusted to pH 2.2 with trifluoroacetic acid (TFA) and trace-enriched by pumping directly on to a 100×4.6 mm RP-300 column (Brownlee) attached to a Beckman System Gold HPLC system and equilibrated in 0.1% TFA. The column was developed with a steep gradient of acetonitrile in 0.1% TFA ($1.1\% \text{ min}^{-1}$) and mesoderm-inducing activity eluted at $\sim 32\%$ acetonitrile. XTC-MIF was further purified by diluting the active fractions fourfold with 0.1% TFA and applying the sample to the same column followed by elution with a shallower gradient of acetonitrile ($0.18\% \text{ min}^{-1}$). Mesoderm-inducing activity again eluted at approximately 32% acetonitrile. Active fractions were pooled and an aliquot representing half the material was diluted and applied to a 100×2.1 mm RP-300 column (Brownlee) which was run at 0.2 ml min^{-1} . The elution profile from this column is shown here. Buffer A was 0.1% TFA and buffer B was 80% acetonitrile in 0.1% TFA. Mesoderm-inducing activity eluted at the position indicated by the bar, coincident with a single ultraviolet-absorbing peak. When aliquots of this peak were analysed by polyacrylamide gel



electrophoresis (inset) a single major band was observed under both reducing (R) and non-reducing (NR) conditions. The minor band visible in the non-reducing lane at $\sim 40,000 \text{ M}$, is an artefact that was not present when the sample was analysed under non-reducing conditions on other occasions (data not shown). Positions of relative molecular mass markers are shown ($\times 10^{-3}$). A sample of this material was used for N-terminal amino-acid sequencing (Fig. 3).

homologue of activin A and not, as has been suggested⁶, of TGF- $\beta 2$. To confirm this we carried out three biological assays, one specific to TGF- β and two specific to activins. First, we found that XTC-MIF is at least 300 times less effective than TGF- $\beta 2$ in inhibiting the growth of CCL-64 mink lung epithelial cells (Fig. 4a), a line whose growth is extremely sensitive to inhibition by TGF- β (ref. 15). Recombinant bovine activin A and murine activin A purified from the WEHI-3B cell line are also ineffective in this assay (K.V.N. and D.H., unpublished

results; R. M. Albano *et al.*, manuscript in preparation). In the second experiment we found that XTC-MIF, like recombinant bovine activin A, specifically and in a dose-dependent fashion stimulates the release of follicle-stimulating hormone, but not luteinizing hormone (data not shown), from pituitary cells (Fig. 4b). The 50% effective dose (ED_{50}) of XTC-MIF in this assay is $\sim 2.5 \text{ ng ml}^{-1}$, which is similar to the reported ED_{50} for porcine activin A (refs 11, 12), recombinant bovine activin A (K.V.N. and D.H., unpublished results), recombinant human activins A and B (ref. 16) and murine activin A purified from the WEHI cell line (R. M. Albano *et al.*, in preparation). Finally, our results showed that XTC-MIF, like recombinant activin A, but unlike TGF- $\beta 1$ and TGF- $\beta 2$, causes K562 cells to terminally differentiate into haemoglobin-synthesizing cells (Table 1). The specific activity of XTC-MIF in this assay is similar to that of murine activin A (R. M. Albano *et al.*, in preparation). Thus XTC-MIF not only shares N-terminal amino-acid sequence with activin A

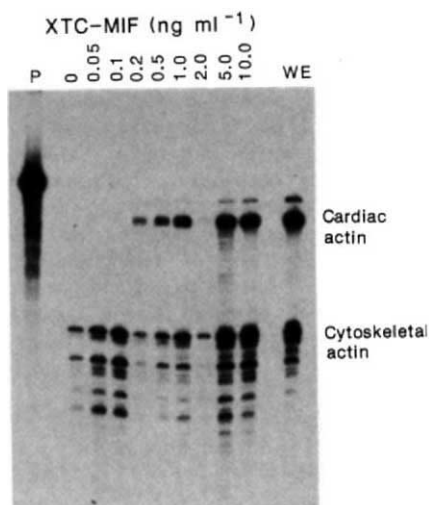


FIG. 2 XTC-MIF induces cardiac actin from mid-blastula *Xenopus* animal caps at concentrations of 0.2 ng ml^{-1} and above.

METHODS. Groups of three stage-8 (ref. 23) *Xenopus* animal pole regions were exposed to the indicated concentrations of XTC-MIF in the presence of 1 mg ml^{-1} bovine serum albumin in 75% normal amphibian medium²⁴. Samples were frozen at stage 25 and analysed by RNase protection using a cardiac actin probe provided by Dr T. J. Mohun (NIMR, Mill Hill). This probe is protected for about 280 nucleotides by cardiac actin mRNA (which is also expressed strongly in somite muscle) and for about 130 nucleotides by cytoskeletal actin mRNA. Each lane represents a third of the total sample; that is, RNA from one animal cap. WE represents RNA from one fifth of a stage-25 embryo and P shows the probe used in this experiment.

	XTC-MIF	Act A	Act B	Inhibin- α	TGF- $\beta 2$	Vg1	DPP
1	G (+d)	G	G	H	A	S	H
2	L	L	L	A	L	Y	S
3	e	E	E	V	D	S	L
4	X	C	C	G	A	K	Y
5	D	D	D	G	A	L	V
6	G	G	G	F	Y	P	D
7	K	K	R	M	C	F	F
8	V	V	T	R	F	T	S
9	N	N	N	R	R	A	D
10	I	I	L	G	N	S	V

FIG. 3 N-terminal amino acid sequences (using the one-letter code) of XTC-MIF, human activins (Act) A and B (refs 25, 26), bovine inhibin- α (ref. 27), *Xenopus* TGF- $\beta 2$ (ref. 28), *Xenopus* Vg1 (ref. 7) and the shortest protein likely to be encoded by the *Drosophila* decapentaplegic complex (DPP) (ref. 29). X in the sequence of XTC-MIF represents an undetermined residue in a position corresponding to cysteine in activins A and B. The sequence of XTC-MIF is identical to human activin A but differs from all the other above proteins and also from TGF- $\beta 1$, 3, 4 and 5 (see refs 28, 30–33).

METHODS. XTC-MIF (24 pmol) from microbore reverse-phase HPLC (Fig. 1) was subjected to Edman degradation on an Applied Biosystems 477A pulsed liquid protein sequencer. The phenylthiohydantoin (PTH) amino acid derivatives were identified by reverse-phase HPLC with an on-line 120A PTH amino acid analyser. Residues in lowercase were either minor components (d at position 1) or indicate that identification was tentative (e at position 3).

but also shows similar biological properties. We conclude that XTC-MIF is the *Xenopus* homologue of mammalian activin A. Mammalian activin A has recently been shown to have mesoderm-inducing activity in *Xenopus* embryos^{17,18}.

Our results show that a molecule that has a role in the adult organism in promoting the specific release of follicle-stimulating hormone¹⁹, and which also modulates the proliferation of multipotential and erythroid haematopoietic progenitor cells²⁰, thus indicating that it is important in erythroid differentiation, may also have a role in early development in the formation of the mesoderm. It may become a common observation that the molecules that regulate or maintain the adult organism are those

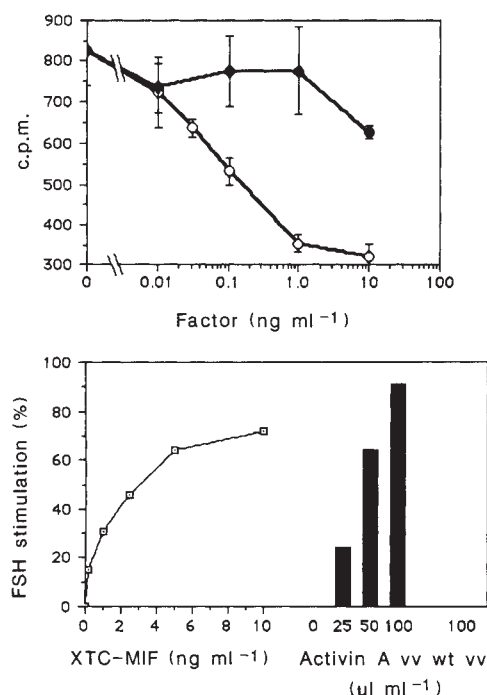


FIG. 4 *a*, XTC-MIF (●), unlike TGF-β2 (○), does not inhibit the growth of CCL-64 mink lung epithelial cells. *b*, XTC-MIF, like recombinant bovine activin A, promotes the release of follicle-stimulating hormone (FSH) from pituitary cells.

METHODS. *a*, The indicated concentrations of XTC-MIF and TGF-β2 were tested for their effects on the growth of CCL-64 cells as described³⁴. Subconfluent CCL-64 cells were trypsinized, washed, and seeded at 5×10^5 cells per 0.5 ml in 24-well Nunclon dishes in 0.2% fetal bovine serum in DMEM. After 1 h, XTC-MIF or TGF-β2 (R&D systems) samples were added and 24 h later the cells were pulsed with 0.25 μCi of [methyl-³H]thymidine. Incorporation of radioactivity into acid-insoluble material was determined 1 h later. Determinations were made in triplicate; values shown are means and bars represent standard errors. *b*, Dispersed rat anterior pituitary cells (2.5×10^5 cells) were cultured at 37 °C in 1 ml DMEM supplemented with 5% horse serum, 2.5% fetal bovine serum, 1% glutamine and 1% non-essential amino acids. After two days the medium was replaced with 1 ml of medium containing the indicated concentrations of XTC-MIF, or recombinant bovine activin A expressed in vaccinia virus (Activin A vv), or a wild-type vaccinia virus control (wt vv). For activin A, the figure shows the volume of medium from HeLa cells infected with bovine activin A recombinant vaccinia virus (D.H. *et al.*, submitted) that was added to the test anterior pituitary cells. By comparison with results obtained with purified murine activin A (R. M. Albano *et al.*, in preparation), we estimate that this medium contains 200–400 ng ml⁻¹ activin A. Incubation of test cells continued for three days and then appropriately diluted culture media were assayed for FSH and luteinizing hormone by double-antibody methods using NIAMDD rat pituitary gonadotropin reagents supplied by the pituitary agency of the National Institutes of Health. All assays were done in triplicate and the figure shows the mean values expressed as stimulation over basal FSH levels obtained with appropriate controls. A reference standard preparation of bovine follicular fluid, a source of inhibin activity, inhibited the release of FSH in a dose-dependent fashion with a maximum value of 50% in the assay shown here.

TABLE 1 XTC-MIF stimulates the differentiation of K562 cells

Sample	Titre (EDF-U ml ⁻¹)
XTC-MIF (200 ng ml ⁻¹)	320
Activin A (expressed in vaccinia virus)	1,280
Wild-type vaccinia virus	NS
TGF-β1 (200 ng ml ⁻¹)	NS
TGF-β2 (200 ng ml ⁻¹)	NS
BSA-HCI	NS

Induction of erythroid differentiation of erythroleukaemia cells by XTC-MIF. Test samples were subjected to twofold serial dilution in RPMI-1640 medium buffered with 25 mM HEPES and supplemented with 10% fetal bovine serum. Dilutions were made in a 96-well microtitre plate with final volumes of 100 μl. K562 cells (10^4), in a volume of 100 μl, were then added to each well and the cells were incubated for 3–5 days at 37 °C before being stained with benzidine. Bovine haemin (Sigma type I) was used as a positive control. The EDF-titre (expressed as K562 EDF-units (EDF-U) ml⁻¹) is defined as the reciprocal of the highest dilution of the sample in 1 ml at which differentiation of the K562 cells is visible compared with complete RPMI-1640 medium. Addition of 100 μM haemin reproducibly induced differentiation of 30% of the target cells, whereas spontaneous differentiation accounted for only 5% (taken here as non-significant, NS). We estimate that our recombinant activin A preparation contains 200–400 ng ml⁻¹ of the protein (see legend to Fig. 4b).

that had previously been used transiently in its development. Our data also raise the possibility that the inhibins, which inhibit both release of follicle-stimulating hormone and also (albeit with lower specific activity) erythroid differentiation¹⁹, also inhibit mesoderm induction; there is evidence that *Xenopus* embryos produce inhibitors of mesoderm formation^{21,22}. We are going on to investigate this and to study the potential roles of activins and inhibins in early mammalian development. □

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Activin-like factor from a *Xenopus laevis* cell line responsible for mesoderm induction

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INDUCTION of mesoderm during early amphibian embryogenesis can be mimicked *in vitro* by adding growth factors, including heparin-binding and type-beta transforming growth factors (TGF- β), to isolated ectoderm explants from *Xenopus laevis* embryos¹⁻⁵. Although the mesoderm-inducing factor (MIF) from *X. laevis* XTC cells (XTC-MIF)^{6,7} has properties similar to TGF- β , this factor is still unidentified. Recently, we obtained a number of homogeneous cell lines from the heterogeneous XTC population, which differ in their MIF production⁸. Only one, XTC-GTX-11, produced MIF, although it was similar to the rest of the clones in its production of known growth factors, including TGF- β activity⁸. This observation, together with the identification of activin A as a potent MIF (ref. 9) led us to study the parallel activities of MIF and activin. Here we report an analysis of activin-like activity from XTC cells and some of the XTC clones, including XTC-GTX-11. There is a clear consistent correlation between MIF activity and presence of activin activity, indicating that XTC-MIF is the *Xenopus* homologue of mammalian activin.

Analysis of conditioned media from XTC and various XTC-derived clones has shown that, apart from the heterogeneous but mainly tetraploid XTC cell line, only the diploid clone XTC-GTX-11 produced mesoderm-inducing activity⁸. The mesodermal tissues induced by these media in stage-10 *Xenopus* ectoderm explants were compared with mesoderm derivatives

induced by recombinant activin A. This activin A was prepared in HeLa cells infected with vaccinia virus (vv), carrying complementary DNA for the inhibin- β_A (activin A) subunit (D.H. *et al.*, submitted). As shown in Fig. 1 the activin preparation contains 60–70% mature activin A (relative molecular mass (M_r) 24,000). In addition, three other activin polypeptides are present, representing the M_r 110,000 (110K) homodimeric precursor form of activin A (pro- β_A monomer, 54K), a 67K heterodimeric protein composed of one proactivin A subunit (54K) and a mature activin A subunit (14K), and the pro-part resulting from cleavage of the precursor (43K).

As shown in Fig. 2, the vv-activin A preparation has strong MIF capacity when tested at a 1:10 dilution. After incubation for 24 h all explants changed their morphology from spherical to elongated structures, as already described for mesoderm induction by other MIFs^{1,6}. Histological examination showed that the explants treated with activin A differentiated to dorsal mesoderm, including notochord and muscle (Fig. 2b, c). Neural tissue was also formed as a result of secondary induction by the mesoderm. Media from uninfected cells or cells infected with wild-type vaccinia virus did not induce mesoderm (Fig. 2a). The mesoderm derivatives induced by activin A closely resemble those induced by MIF produced by XTC and by XTC-GTX-11. Very strong induction of dorsal mesoderm was observed, however, only on heating or acidifying conditioned medium from the uncloned XTC line, suggesting the presence of a heat- and acid-labile inhibitor of mesoderm induction^{6,8}. In contrast, untreated conditioned medium from XTC-GTX-11 contained very strong MIF activity, which could not be increased further by heat or acid treatment⁸. As it has been shown previously that this MIF activity is unrelated to TGF- β and fibroblast growth factor (FGF)⁸, activin could be the mesoderm inducer secreted by *Xenopus* cell lines.

The presence of activin activity in conditioned medium from XTC-derived clones was determined in two assays based on the fact that activin A is identical to erythroid differentiation factor (EDF)¹⁰ and to follicle-stimulating hormone (FSH)-releasing protein (FRP)^{11,12}. Media from uncloned XTC and from XTC-GTX-11 were tested, with media from control clones (XTC-GTX-7, -9 and -13) that did not induce mesoderm formation. Of the untreated media, only XTC-GTX-11 showed stimulation of FSH release, which remained unchanged by heat treatment (Table 1). In contrast, heat treatment markedly increased the FSH-releasing capacity of conditioned medium from uncloned XTC cells, in parallel with the heat-induced increase in MIF

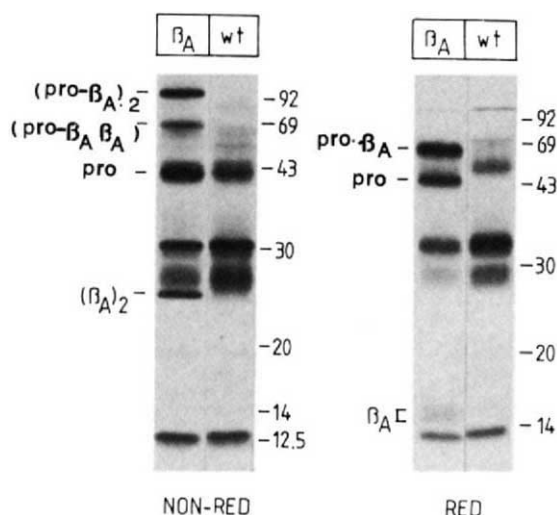


FIG. 1 Comparison of radiolabelled secretory proteins from HeLa cells infected with recombinant (activin A) vaccinia virus β_A , or wild-type (wt) virus. Pro- β_A , proactivin (residues 1–397); β_A , mature activin (282–397); pro, N-terminal part of pro- β_A (1–281); (pro- β_A)₂ and (β_A)₂ refer to disulphide-linked homodimeric forms, pro β_A - β_A to the disulphide-linked heterodimeric protein containing the subunits indicated. NON-RED, non-reduced, and RED, reduced samples; M, M_r marker proteins. The complete description of the biosynthesis and intracellular cleavage of proactivin A will be described elsewhere (D.H. *et al.*, submitted).

METHODS. Subconfluent monolayers of cells were infected with virus (multiplicity of infection 5–10) for 1 h at 24 °C. The inoculum was removed and the infected cells were incubated for 15 h at 37 °C in 1 ml DMEM with 20 μ g BSA ml⁻¹. Medium from the overnight infection was removed, filtered through a low-protein-absorbing, 0.22- μ m filter and stored frozen until assay (Tables 1 and 2). Cells were washed twice with methionine-free MEM Earle's salts)–20 mM HEPES before starvation in this medium for 1 h at 37 °C. They were pulse-labelled for 30 min by adding medium containing 50 μ Ci [³⁵S]methionine ml⁻¹, and then chased in DMEM plus 20 μ g BSA ml⁻¹ which contained the 10-fold molar excess of cold methionine that is normally present in this medium. After 4 h at 37 °C, the medium was collected, centrifuged and the supernatant frozen. Protein samples were prepared for electrophoresis as follows: BSA (50 μ g ml⁻¹) was added to the media and the proteins precipitated with 10% trichloroacetic acid. The protein pellet was neutralized and solubilized in 0.5 M Trizma (Sigma)-6.25% sodium dodecyl sulphate (SDS), diluted 4.5-fold with water and adjusted to SDS-polyacrylamide gel electrophoresis conditions (15% gels)¹⁴.

TABLE 1 Effects of conditioned media from XTC cells and clonal XTC derivatives on the specific release of FSH from cultured pituitary cells

Sample	No treatment	Heat treated
XTC medium	NS	49%
XTC-GTX-11 medium	58%	64%
XTC-GTX-7 medium	NS	NS
XTC-GTX-9 medium	NS	NS
XTC-GTX-13 medium	NS	NS
HeLa vv- β_7 (activin A)	143%	ND
HeLa wild-type control	NS	ND
HeLa mock (not infected)	NS	ND
BFF 2.5 μ l	-9%*	ND
BFF 5 μ l	-28%	ND
BFF 10 μ l	-52%	ND
BFF 20 μ l	-50%	ND
TGF- β_2 † 0.6 ng	19%	ND
TGF- β_2 1.25 ng	17%	ND
TGF- β_2 2.5 ng	NS	ND
TGF- β_2 5 ng	11%	ND

* Inhibition of FSH release by bovine follicular fluid (BFF), a predominant source of inhibin for this assay.

† Measured with another preparation of pituitary cells.

The media were tested in triplicate both before and after heat treatment (5 min at 95 °C). Figures are mean values for percentage stimulation of FSH release as compared with basal FSH levels in the presence of phosphate-buffered saline. NS, Non-significant, <7.5%; ND, not determined.

XTC cells were supplied by Dr E. A. Jones (University of Warwick, UK). XTC subclones were selected on the basis of ability to proliferate in soft agar and cultured as described⁸. Cells were cultured at 25 °C under air, in 67% Leibovitz L15 medium with 10% fetal calf serum^{6,7}. The medium was also supplemented with 2 mM glutamine, 12.5 IU penicillin and 12.5 μ g streptomycin ml⁻¹. Conditioned medium was obtained from XTC cells, which were first grown to confluence in serum-containing medium. The cells were then incubated in serum-free 67% L15 medium, which was discarded after 5 h. The cells were then cultured 24 h in fresh serum-free medium. Conditioned medium was collected and the serum-free medium refreshed every 24–48 h, for periods of up to one month. The conditioned medium was stored frozen. Dispersed rat anterior pituitary cells (2.5×10^5 cells) were cultured at 37 °C in 1 ml DMEM, supplemented with 5% horse serum, 2.5% fetal bovine serum, 1% glutamine and 1% non-essential amino acids. After 2 days in culture, the medium was replaced with 1 ml XTC-cell medium or medium containing diluted vaccinia material (1:10, so 100 μ l medium from mock-infected or wild-type or bovine activin vaccinia virus (vv) β_7 -infected cells were added). A standard reference preparation of BFF inhibited FSH release in a dose-dependent fashion with a maximum value of ~50% in the assay shown here. Samples were incubated for 3 days and appropriately diluted culture medium was then assayed for FSH and LH by double-antibody methods using NIAMDD rat pituitary gonadotropin reagents supplied by the pituitary agency of the NIH. The samples tested did not significantly influence the release of LH from the pituitary cells (data not shown). Pure porcine TGF- β_2 never showed a dose-dependent FSH-stimulation and never scored more than 20% stimulation.

TABLE 2 Effects of conditioned media from XTC cells and clonal XTC derivatives on the differentiation of the erythroleukaemia cell line K562

Sample	No treatment (EDF units ml ⁻¹)	Heat treatment (EDF units ml ⁻¹)
XTC	NS	20
XTC-GTX-11 medium	NS	320
XTC-GTX-7 medium	NS	NS
XTC-GTX-9 medium	NS	NS
XTC-GTX-13 medium	NS	NS
HeLa vv- β_7 (activin A)	640	ND
HeLa wild-type control	NS	ND
HeLa mock (not infected)	NS	ND

NS, Only spontaneous differentiation; ND, not determined.

All media were tested both before and after heat treatment (5 min at 95 °C). Differentiation of K562 cells (ATCC CCL-243) was measured by the production of haemoglobin. Twofold serial dilutions of the samples to be tested (in 100 μ l RPMI-1640 medium buffered with 25 mM HEPES and supplemented with 10% fetal bovine serum) were made in a 96-well microtitre plate; 10^4 target cells (in 100 μ l) were then added to each well. Bovine haemin (type I, Sigma) was used as a positive control. Cells were incubated for 4 days at 37 °C and then stained with benzidine. The EDF titre (expressed as K562 EDF units ml⁻¹) is defined as the reciprocal of the highest dilution of the sample in 1 ml at which differentiation of the K562 cells is still visible compared with complete RPMI-1640 medium. Addition of 100 μ M haemin reproducibly induced differentiation of 30% of the target cells, whereas spontaneous differentiation accounted for only ~7% in the assay shown here (indicated as NS).

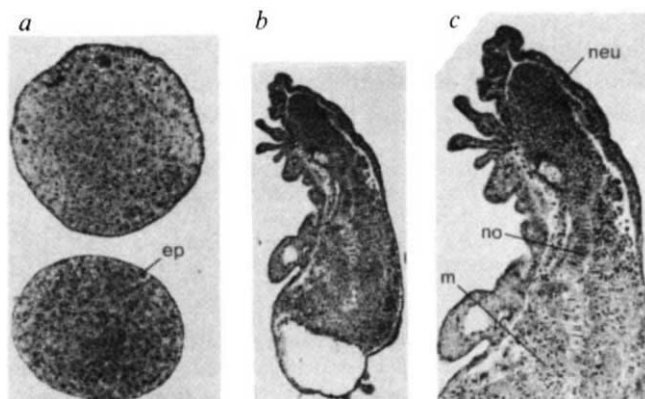


FIG. 2 Effects of medium from HeLa cells infected with a, wild-type vaccinia virus, and b, recombinant vaccinia virus, on mesoderm induction of stage-10 *Xenopus laevis* ectodermal explants; c is a magnification of b. a, Untreated controls remain spherical, and differentiate to epidermis (ep). The same result was obtained for media from uninfected HeLa cells and cells infected with wild-type vaccinia virus. b, c, Explants treated with activin A always become strongly elongated and show extensive differentiation to notochord (no), and to muscle (m), and also neural differentiation (neu), as well as epidermal differentiation.

METHODS. Embryos of *Xenopus laevis* were dejellied chemically using 2% cysteine-HCl (pH 7.8–8.0) and washed several times. When the embryos had reached stage 10 (ref. 15), the vitelline membrane was removed with forceps and the animal (ectodermal) cap dissected and transferred, with blastocoel facing up, to Flickinger medium containing the factor to be tested. Recombinant activin A and control media (media from uninfected HeLa cells or cells infected with wild-type vaccinia virus) were tested at a 1:10 dilution in Flickinger medium¹⁶. After incubation for 18–20 h, the explants were cultured at 21–23 °C with three changes in Flickinger medium, until control embryos reached stage 40; all embryos were then fixed in Smith's fixative (2.5% acetic acid, 0.5% K₂Cr₂O₇, 4% formaldehyde) for 4 h. After 2 h of washing in tap water, the fixed explants were dehydrated, embedded in paraffin, sectioned, stained with haematoxylin–eosin and examined for the presence of mesodermal structures. Twenty to twenty-five explants were tested for each treatment in each of four independent experiments (activin A and control).

activity. The inhibitory action of inhibin, the stimulatory action of recombinant activin A and the effect of TGF- β_2 are shown for comparison.

As shown in Table 2, none of the untreated conditioned media seemed to contain EDF activity, whereas an EDF activity of 640 units ml⁻¹ was detected for recombinant activin A. Heat treatment increased the EDF activity of XTC-GTX-11-conditioned medium to 320 units ml⁻¹, however, whereas there was an increase to 20 units ml⁻¹ for XTC medium. The lack of EDF activity in untreated XTC-GTX-11 medium may be explained by the presence of a heat-labile differentiation-inhibiting activity. The results obtained so far show that a clear correlation exists between the presence of activin and MIF activity. For this reason it is very likely that XTC-MIF is an activin-like factor¹³.

Preliminary measurements using these bioassays have shown that blastula (stage 8) *Xenopus* embryos contain a detectable level of activin. These results together with localization and functional studies of other amphibian MIFs will contribute to

unravelling the signals that mediate the complex process of mesoderm induction *in vivo*. □

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Localization of the human GM-CSF receptor gene to the X–Y pseudoautosomal region

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MAMMALIAN sex chromosomes share a small terminal region of homologous DNA sequences, which pair and recombine during male meiosis (reviewed in ref. 1). Alleles in this region can be exchanged between X and Y chromosomes and are therefore inherited as if autosomal. Genes from this so-called pseudoautosomal region (PAR) are present in two doses in both males and females, and escape inactivation of the X chromosome in females. Indirect evidence suggests that there must be several pseudoautosomal genes, and several candidates have been proposed. Until now, the only gene that has been unequivocally located in the PAR is *MIC2*, which encodes a cell-surface antigen of unknown function^{2,3}. We now report the localization of a gene of known function to this region—the gene for the receptor of the haemopoietic regulator, granulocyte-macrophage colony stimulating factor. The chromosomal localization of this gene may be important in understanding the generation of M2 acute myeloid leukaemia.

Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates the proliferation, differentiation and functional activation of granulocytes and macrophages⁴. We have recently cloned a complementary DNA encoding the human cellular receptor for GM-CSF (ref. 5). The human GM-CSF receptor (GM-R) is encoded by a gene extending over about 50 kilobases (kb) of DNA (N.M.G., unpublished results). To gain insight into the possible genetic relationship of the GM-R gene to genes for other haemopoietic regulators and their receptors, and to

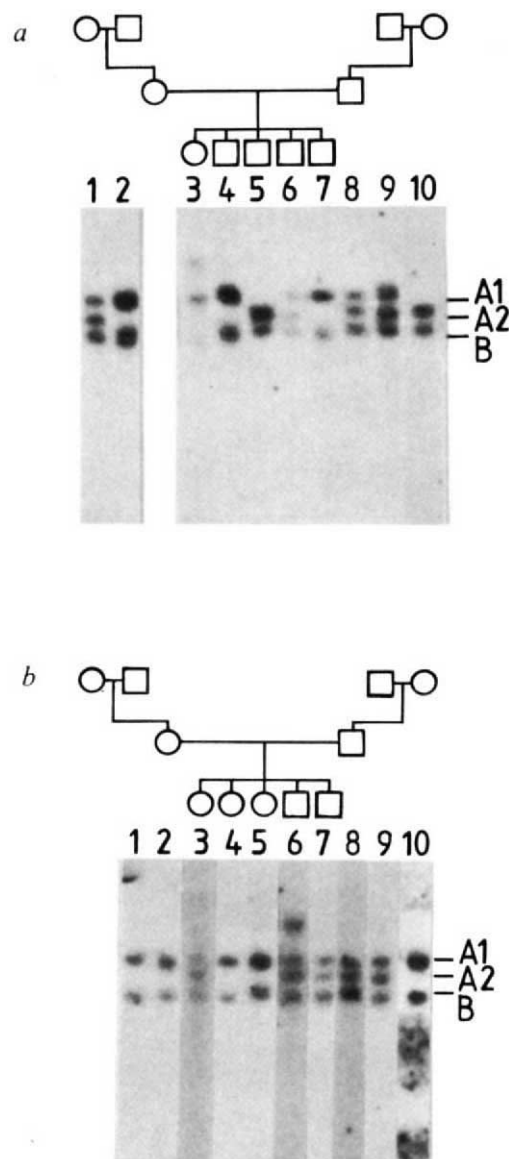


FIG. 1 Pseudoautosomal inheritance in 2 three-generation families of *Hind*III restriction fragment length polymorphisms (RFLPs) of the GM-CSF receptor locus. The pedigree for each family is given above each set of data (squares, males; circles, females). In Utah family 984 (a) the father (lane 8) has inherited allele A2 of fragment A from his mother (lane 10) and thus A2 is X-located, whereas A1, inherited from his father (lane 9), must be Y-located. Information was not obtained from the mother. She was presumed to be heterozygous in order to explain the genotypes of the offspring; heterozygosity would be compatible with the genotypes of the maternal grandparents (lanes 1 and 2). In Utah family 985 (b) the father (lane 8) has inherited allele A1 from his mother (lane 10) and thus A1 is X-located, whereas A2, inherited from his father (lane 9), must be Y-located. The higher relative molecular mass bands in lane 3 (a) and lane 6 (b) are partial digestion products. The Utah DNA samples³⁴ were: (a) 7056 (lane 1), 7022 (lane 2), 7053 (lane 3), 7008 (lane 4), 7040 (lane 5), 7342 (lane 6), 7027 (lane 7), 7029 (lane 8), 6994 (lane 9), 7000 (lane 10); (b) 6993 (lane 1), 6991 (lane 2), 7044 (lane 3), 7012 (lane 4), 7344 (lane 5), 7021 (lane 6), 7020 (lane 7), 7048 (lane 8), 7034 (lane 9), 7055 (lane 10).

METHODS. Aliquots (10 µg) of high relative molecular mass genomic DNA were digested with *Hind*III, electrophoresed on 0.6% agarose gels and transferred to nitrocellulose. Conditions for hybridization and washing were as described previously⁵. The hybridization probe was the gel-purified 786 base pair *Kpn*–*Eco*RI cDNA fragment spanning the 3' end of the GM-CSF receptor coding region derived from clone pGMR138 (ref. 5) radiolabelled to a specific activity of approximately 2.4×10^8 c.p.m. µg⁻¹ by nick-translation and included in the hybridizations at approximately 2×10^7 c.p.m. ml⁻¹.

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TABLE 1 Assignment of the GM-CSF receptor gene to the X chromosome

Hybrid cell line	Human chromosome content	Hybridization	Ref.
CY2	der(X)t(X;16)(q26;q24)	+	36
CY3	der(16)t(X;16)(q26;q24)	—	36
CY4	der(13)t(13;16)	—	30
CY5	der(10)t(10;16), 22	—	36
CY6	der(10)t(10;16), 8	—	29
CY7	der(3)t(3;16), 10, 12	—	36
CY12	der(16)t(12;16), 2, 7, 8, 12, 21	—	31
CY13	der(16)t(1;16), 3, 11, 14, 17, 20, 21, 22	—	36
CY15	del(16)(p12), 11, 14, 17, 21	—	31
CY18	16	—	35
CY34	der(X)t(X;5)(q27;q32), 4, 7, ?	+	32
4.12	X	+	33
PeCH-N	der(X)t(X;21)(q27;q11), ?	+	32

The GM-CSF receptor probe (Fig. 1) was radiolabelled and hybridized to a nylon membrane filter containing *Taq*I-digested DNA from a panel of mouse-human hybrid somatic cell lines, using standard methods. Hybridization to the human GM-CSF receptor probe is indicated by '+'. Note that the human GM-CSF receptor probe does not cross-hybridize to a murine counterpart on genomic Southern blots (N.M.G., unpublished results). '?' indicates that there may be other human autosomes present. Note cell line 4.12 to which the human GM-R probe hybridizes and in which the only human chromosome is the X. Because cell lines CY2 and CY3 contain reciprocal portions of the X chromosome, hybridization of the GM-R probe to CY2 but not CY3 localizes this gene to the region Xpter-q26.

other genes implicated in growth control and tumorigenesis, we decided to determine its chromosomal location. Southern blot analysis of a panel of mouse-human somatic cell hybrids, which retain various human chromosomes, indicated that the GM-R gene is on the X chromosome (see Table 1 for details). The possibility that an allele of the GM-R locus also maps to the Y chromosome was suggested by the observation that many DNA samples from normal males carry two alleles of this gene. For example, in *Hind*III digests of genomic DNA (Fig. 1), a cDNA fragment corresponding to the 3' half of the GM-R coding region detects an invariant ~11 kb fragment (B) and a polymorphic fragment of ~14–19 kb (A). At least six slightly different sized alleles of fragment A have been detected. Among 65 normal individuals, 23 females and 20 males were homozygous for an

allele of fragment A, whereas 8 females and 14 males were heterozygous (N.M.G., unpublished results, and Fig. 1). To confirm the assignment of this gene and to map the locus regionally, we performed *in situ* hybridization to metaphase chromosomes (Fig. 2). In this way the GM-R gene was mapped to the tip of the short arm of the X chromosome and the short arm of the Y chromosome, with the most likely localization Xp21-pter and Ypter-p11.2.

Localization of the GM-R gene to the tip of the X and Y chromosomes is consistent with it being pseudoautosomal. However, formal identification of it being within this region required demonstration of exchange between X and Y chromosomes during male meiosis. Therefore, the segregation of the GM-R locus with respect to sexual phenotype was investigated in three 3-generation families with large sibships. For one such family (Fig. 1a) of five offspring (lanes 3–7), the daughter (lane 3) had inherited the allele of the GM-R gene from the paternal Y chromosome (A1) and one son (lane 5) had inherited the allele from the paternal X chromosome (A2). In two sons (lanes 4 and 7) no exchange had taken place and a further son (track 6) was uninformative, being heterozygous. Figure 1b shows the results for a second family in which one daughter (lane 3) had inherited the allele from the paternal Y chromosome (A2). In the other four offspring no exchange had taken place. In a third family (not shown) with five informative offspring, no recombinants were observed. Therefore in a total of 14 informative male meioses, 3 recombination events were observed. The relatively high frequency with which the GM-R locus recombines between the X and Y chromosomes (~20%) is similar to the frequencies observed for several anonymous pseudoautosomal loci (14–50%; ref. 6) and contrasts with the low frequency of *MIC2* recombination (~2.5%; ref. 2). The *MIC2* locus has been mapped close to the PAR boundary and the higher rate of exchange of the GM-R locus suggests that it maps distal to *MIC2*, probably between the loci *DXYS15* and *DXYS17* (refs 1, 6).

It was long thought that mammalian sex chromosomes have sequences in common, which pair during male meiosis and allow exchange between the X and Y chromosomes^{7–10}. The existence

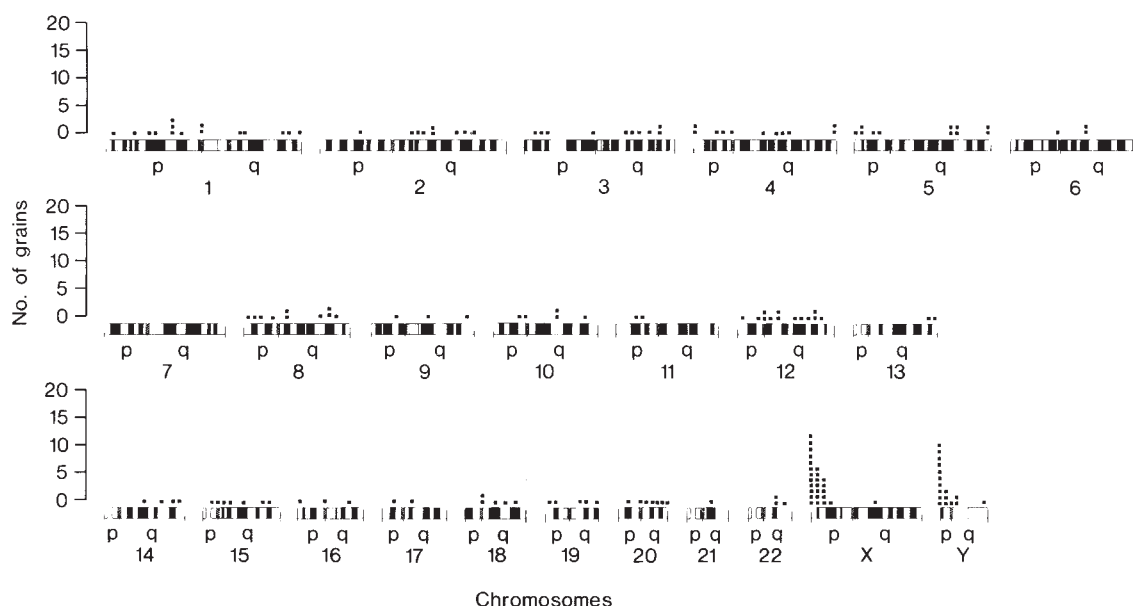


FIG. 2 Distribution of silver grains from 45 metaphases after hybridization with the GM-CSF receptor gene probe. The probe was a pIC20H plasmid containing the *Kpn*I-*Eco*RI fragment of the GM-CSF receptor cDNA (Fig. 1). The probe was radiolabelled by nick-translation with three tritiated nucleotides to a specific activity of 1.1×10^8 c.p.m. μg^{-1} , hybridized to metaphases from two normal males at a concentration of $0.001 \mu\text{g ml}^{-1}$ and exposed

to autoradiographic emulsion for 11 days³⁵. All silver grains touching chromosomes were counted to determine the pattern of hybridization of the probe. Of 177 silver grains over the 45 metaphases, 25 grains (14.1%) were over the region Xp21-pter and 16 grains (9%) were over Ypter-q11.2. A similar result was obtained after hybridization to the chromosomes of the second male (data not shown).

of such a pseudoautosomal region has recently been directly demonstrated, and physical and genetic maps of the human PAR, which encompasses about 2,500 kb, determined¹¹⁻¹⁶. There is much interest in the nature of the genes in this region and indeed several candidates have been proposed, including genes associated with schizophrenia¹⁷, cerebral dominance¹⁸ and Turner's and Klinefelter's syndromes^{9,19}. *MIC2*, which maps just within the PAR, encodes a cell-surface molecule implicated in T-cell adhesion processes^{2,3,20} and a pseudoautosomal locus, *XGR*, has been postulated to regulate the expression of *MIC2* and the Xg blood group locus²¹. The localization of the human GM-R gene to this region is the first example of a gene with known function. It is of interest that the *MIC2* gene product is also a cell-surface molecule, although based on sequence data the *MIC2*-encoded protein and the GM-R are distinct molecules.

Loss of either the X or the Y chromosome is apparent in 25% of acute myeloid leukaemias (AML) of the M2 subtype, compared with only 1-6% in other AML subtypes²², suggestive of the involvement of a 'recessive oncogene' in the genesis of M2 AMLs²³. The presumed gene is likely to be in the PAR, because if it were localized to the portion of the X chromosome not shared with the Y (most of the X), then similar loss of the Y chromosome would not be predicted, and vice versa. That both chromosomes seem to be involved, implicates a gene that is shared between these chromosomes, and, therefore, most likely to be in the PAR. Although the PAR undoubtedly contains several genes that could be implicated, the GM-CSF receptor gene has the appropriate characteristics to be involved in the generation of AML. Loss or inactivation of both copies of this gene in a myeloid progenitor cell would be expected to generate a clone of cells unable to respond to GM-CSF, and hence possibly with a relatively undifferentiated phenotype similar to that displayed by M2 AMLs. A significant subfraction of AMLs is indeed unresponsive to GM-CSF²⁴⁻²⁸. □

Hereditary spherocytosis associated with deletion of human erythrocyte ankyrin gene on chromosome 8

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HEREDITARY spherocytosis (HS) is one of the most common hereditary haemolytic anaemias¹. HS red cells from both autosomal dominant and recessive variants are spectrin-deficient^{2,3}, which correlates with the severity of the disease³. Some patients with recessive HS have a mutation in the spectrin α -2 domain (S.L.M. *et al.*, unpublished observations), and a few dominant HS patients have an unstable β -spectrin that is easily oxidized⁴, which damages the protein 4.1 binding site and weakens spectrin-actin interactions^{5,6}. In most patients, however, the cause of spectrin deficiency is unknown. The α - and β -spectrin loci are on chromosomes 1 and 14 respectively⁷⁻⁹. The only other genetic locus for HS is *SPH2*, on the short arm of chromosome 8 (8p11)¹⁰⁻¹⁴. This does not correspond to any of the known loci of genes for red cell membrane proteins including protein 4.1 (1p36.2-p34)¹⁵, the anion exchange protein (AE1, band 3; 17q21-qter)^{16,17}, glycophorin C (2q14-q21)¹⁸, and β -actin (7pter-q22)¹⁹. Human erythrocyte ankyrin, which links β -spectrin to the anion exchange protein, has recently been cloned^{20,21}. We now show that the ankyrin gene maps to chromosome 8p11.2, and that one copy is missing from DNA of two unrelated children with severe HS and heterozygous deletions of chromosome 8 (del(8)(p11-p21.1)). Affected red cells are also ankyrin-deficient. The data suggest that defects or deficiency or ankyrin are responsible for HS at the *SPH2* locus.

To localize the ankyrin gene we hybridized²² plasmid pAnk-1B, a 1,405 base pair (bp) clone that codes for the C-terminal regulatory domain (less a 486-bp alternatively spliced sequence) and 407 bp of 3' untranslated sequence²⁰, to flow-sorted human chromosomes. Hybridization occurred to chromosome 8 (Fig. 1). Identical results were obtained in two other experiments using probes that code mostly for residues belonging to the spectrin binding domain (*NcoI*-*Bam*HI (bp 1-371) and *SacI*-*Sma*I (bp 626-1195) fragments of pAnk15 (ref. 20; data not shown)). In addition, when pAnk1B was hybridized to a previously described²² panel of four human-rodent somatic cell hybrids, the pattern of reaction confirmed localization of erythrocyte ankyrin to chromosome 8 (data not shown).

The *SPH2* locus has been mapped to chromosome 8p from studies of families with HS and balanced translocations involving chromosomes 8 and 12 (t(8;12)(p11;p13))^{10,11} or 8 and 3 (t(3;8)(p21;p11))¹² and reports of HS associated with heterozygous interstitial deletions between 8p11.1 (ref. 13) or 8p11.22 (ref. 14) and 8p21.1. The glutathione reductase gene (*GSR*) is located at 8p21.1 (ref. 23), but is not involved in the

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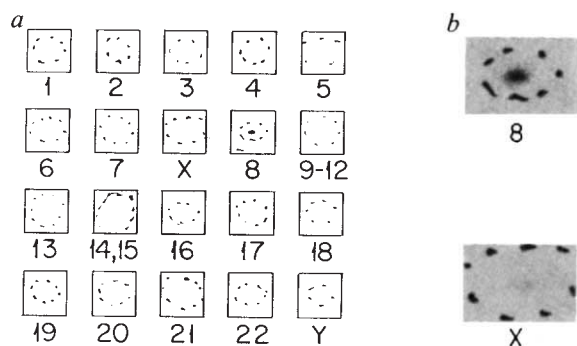


FIG. 1 Localization of human erythrocyte ankyrin gene to chromosome 8. *a*, Autoradiograms of pAnk1B hybridized to spot blots of flow-sorted chromosomes. Each small square contains a single chromosome population or a number of similar sized chromosomes which co-sort (such as 9-12 and 14,15). Chromosome 8 shows a positive signal. Weak hybridization to the X-chromosome population is probably due to occasional missorting of chromosome 8 with X-chromosome samples²². *b*, Enlargement of pools representing chromosomes 8 and X.

METHODS. Metaphase chromosomes were prepared from human lymphoblastoid cells (GM3798), stained with chromomycin A₃ and Hoechst 33258 dyes, and separated²² with a Becton-Dickinson Fluorescence-Activated Cell Sorter IV equipped with dual argon ion lasers. Spot blots were produced by sorting 25,000 individual chromosomes directly onto nylon filters²². Plasmid pAnk1B was hybridized to the flow-sorted chromosomes as described²².

deletions^{13,14}. Thus, *SPH2* lies between the centromere and *GSR*, probably near 8p11.22 (ref. 14). It is of interest that the murine gene for glutathione reductase (*Gr-1*) is also located on chromosome 8 (ref. 24), and the locus for normoblastosis, a form of hereditary spherocytosis characterized by synthesis of an unstable mutant ankyrin, lies between *Gr-1* and the centromere²⁵. This suggests that *SPH2* may represent ankyrin and there may be a previously undescribed area of homology of synteny between mouse and human chromosome 8.

To test this possibility, we hybridized genomic DNA from two unrelated children with HS and chromosome 8 deletions to probes for erythrocyte ankyrin and the anion exchanger AE1 (Fig. 2*a*). One of these children has not previously been reported. His clinical picture is presented briefly (see Fig. 2 legend) and is similar to previous descriptions^{13,14}. In both children, the ratio of ankyrin signal to AE1 signal is about 0.5 (Fig. 2*b*). As AE1 is likely to be a single copy gene²⁶, these gene dosage blots

indicate that one ankyrin gene is deleted in the children and strongly suggest that ankyrin is encoded at the *SPH2* locus.

This was confirmed by directly visualizing the ankyrin gene, using fluorescence *in situ* hybridization coupled with digital imaging microscopy²⁷. This technique permits direct visualization of each copy of the gene on the pair of chromosomes on which it resides. The map position of a gene is determined in terms of the fractional length of the total chromosome relative to the terminus of the short arm (FLpter), which is equivalent to that defined by other cytogenetic techniques²⁷. The ankyrin gene was localized using a biotinylated genomic clone (BT 107), corresponding to the C-terminus of ankyrin, and was detected with fluorescein isothiocyanate (FITC)-conjugated avidin²⁷ (Figs 3*a, e*). The FLpter value of the ankyrin gene on chromosome 8 is 0.27-0.31, corresponding to a cytogenetic map position at band 8p11.2 (Fig. 3*g*).

When lymphoblastoid cells established from one of the

FIG. 2 *a*, Gene dosage blots of *Bam*HI-restricted DNA from a normal individual (N) and two unrelated children (S and H) with HS due to heterozygous deletion of the *SPH2* locus on chromosome 8. The blots were hybridized to probes for ankyrin (ANK, left), the human erythrocyte anion exchanger (AE1, right), or both (centre), and were analyzed by autoradiography. *b*, Relative number of counts hybridized to erythrocyte ankyrin genes versus erythrocyte AE1 genes. Note the two HS patients (S and H) have only about 50% of the normal (N) ankyrin gene dosage, which suggests that each is missing one ankyrin gene. The variance bars indicate the standard deviation of the normal data ($n=6$) and the range of two independent experiments for each of the affected children. Patient S refers to M.S., a previously undescribed 10-year-old white male with an interstitial deletion of 8p11.1-8p21.1. The deletion apparently does not involve the *GSR* gene at 8p21.1 as *GSR* activity of the patient's red cells is normal and comparable to the level in his parents. Associated abnormalities include severe growth and developmental retardation since birth; severe HS (neonatal jaundice, marked spherocytosis, osmotically fragile red cells (incubated osmotic fragility test: 50% haemolysis at 0.73 g dl⁻¹ NaCl, normal 0.53), transfusion dependence postsplenectomy; normal haematocrit and 3.5% reticulocytes (normal 0.8%) postsplenectomy; micropenis (0.5 cm at birth) and bilateral undescended testes with normal serum levels of follicle stimulating hormone and luteinizing hormone; bilateral strabismus and nystagmus; and an aganglionic colon and ileum (Hirschsprung's disease). The child's parents and sister have a normal karyotype, normal red cells and none of the other defects. H refers to B.H., one of two affected sisters described in ref. 13. Her deletion also extends from 8p11.1-8p21.1.

METHODS. DNA was prepared from peripheral blood leukocytes (normals), from splenic tissue (M.S.), or from a lymphoblastoid cell line (GM08014, B.H.). *Bam*HI-restricted genomic DNA (10 µg) was separated on a 0.8% agarose gel, transferred to a Magnagraph nylon membrane (Micron Separations, Westborough, Massachusetts), and hybridized to [α -³²P] dCTP-labelled²² probes (2×10^9 d.p.m. µg⁻¹ DNA) using standard procedures. The ankyrin probe was the 859 bp *Bam*HI fragment of pAnk15 (ref. 20) and the AE1 probe was the 806 bp *Eco*RI-*Xba*I fragment of plasmid pBd3-45B (ref. 17). The data in *b* were obtained using a Betascope Blot Analyzer³³ (Betagen, Waltham, Massachusetts). A 10-h scan was performed using a window (6 mm × 3 mm) aligned with the centre of each of the bands shown. The ankyrin (normal), AE1 (normal and HS), and background windows recorded an average of 16,847, 19,415 and 1,266 counts per 10 h, respectively, under these conditions.

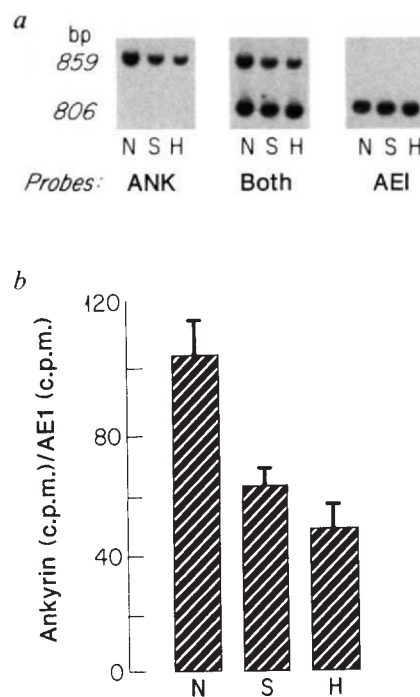


TABLE 1 Erythrocyte membrane protein composition in a patient with HS and heterozygous deletion of chromosome 8p11.1-8p21.1

	Spectrin/AE1	Ankyrin/AE1	4.1/AE1	4.2/AE1	Actin/AE1
Controls (10)	1.056 ± 0.083	0.209 ± 0.033	0.204 ± 0.021	0.201 ± 0.015	0.206 ± 0.025
Patient C.H.	0.830	0.124	0.190	0.148	0.243
(Percentage of controls)	79%	59%	93%	74%	118%

Erythrocyte membranes from controls and patient C.H. (sister of B.H., Fig. 2) were analysed by SDS-PAGE, using 3.5–17% nonlinear gradient gels^{3,35}. The gels were stained with Coomassie blue, and the bands corresponding to spectrin, ankyrin, proteins 4.1 and 4.2, actin and the anion exchange protein (AE1) were excised. The dye was eluted with 25% pyridine and quantified spectrophotometrically (605 nm)³. The controls represent 10 independent experiments, each performed five to eight times. The data from each red cell membrane were normalized to a constant amount of AE1 (to correct for variations in protein loading) and averaged. The results are presented as the mean and standard deviation of these average ratios. For patient C.H., 10 replicate gels were run and processed by the same procedure. The average ratio for each gel band is presented; the standard deviations of these averages varied from 6 to 14%.

children with HS¹³ were examined with this technique, only one hybridization signal was seen per cell, both in metaphase spreads (Fig. 3*b* (arrow), 100% of 25 cells examined) and interphase nuclei (Fig. 3*d*, 90% of 150 cells), indicating that only one copy of the ankyrin gene was present. As expected, control cells showed two hybridization signals (96% of 25 metaphase spreads and 83% of 150 interphase nuclei; Figs 3*a*, *c*). In addition, *in*

situ co-hybridization with probes for both ankyrin and *c-myc* (which maps to chromosome 8q24.1), showed *c-myc* on both chromosomes 8, whereas ankyrin was only detectable on one of the two chromosomes (compare Figs 3*e* and *f*).

Red cells were analyzed from one of the affected children¹³ (Table 1). As expected, the membranes were markedly ankyrin deficient (59% of normal) and showed lesser deficiencies of

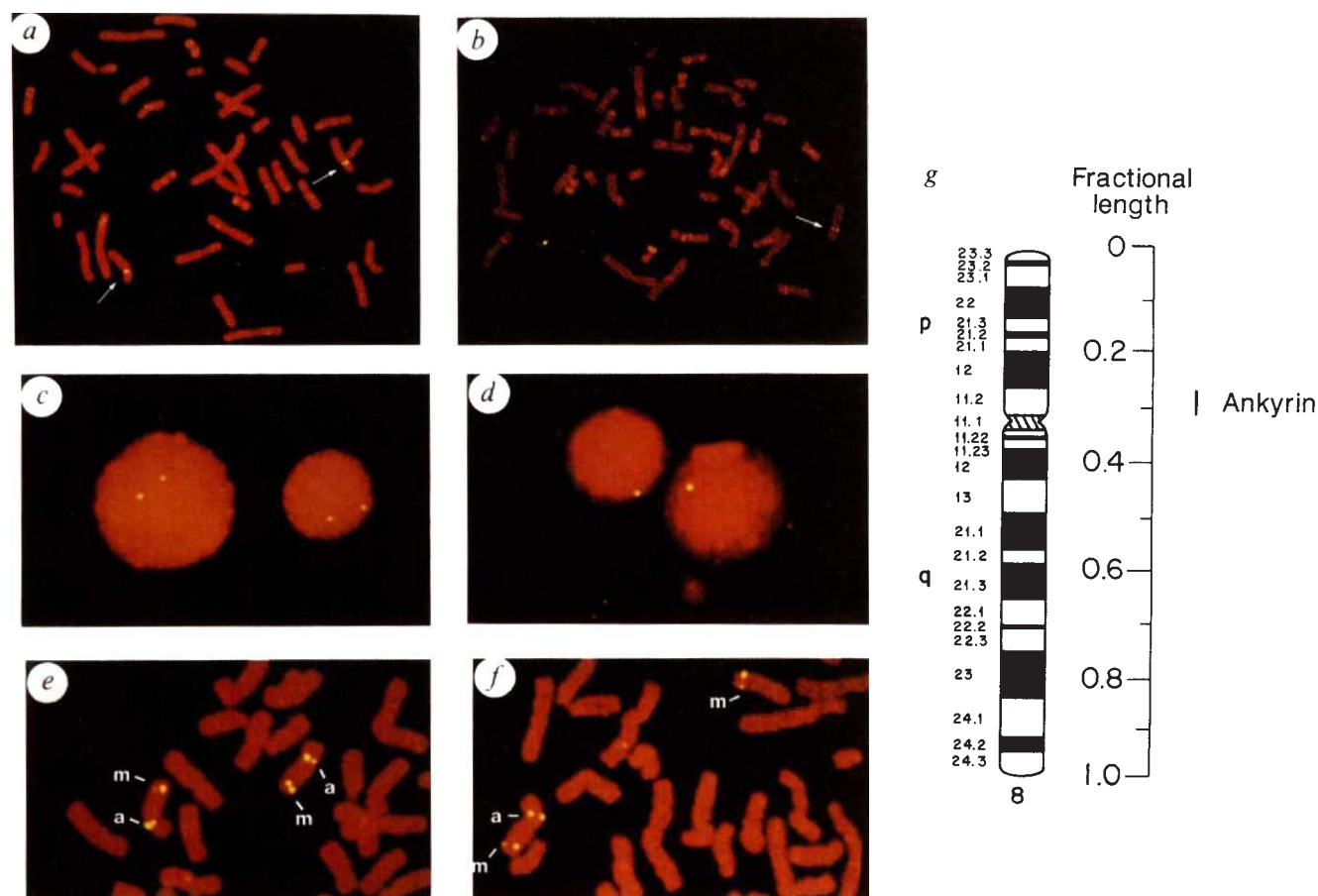


FIG. 3 Demonstration of ankyrin gene deletion in HS by fluorescence *in situ* hybridization. The yellow-green fluorescent signals are viewed against a background of red-stained DNA. *a*, *b*, Metaphase spreads prepared from a normal diploid cell or the HS cell line, respectively. Arrows mark the fluorescent ankyrin genes. Two dots are seen because two sister chromatids are present in metaphase chromosomes. *c*, *d*, Interphase nuclei from the control and HS patient, respectively. Two fluorescent spots represent the two normal ankyrin genes in the control nuclei, but only a single spot is seen in the nuclei of the patient. *e*, *f*, Metaphase spreads of control and HS cells, respectively, co-hybridized with ankyrin (*a*) and *c-myc* (*m*) genomic probes. Both normal chromosomes 8 are labelled by both probes, whereas, both HS chromosomes 8 contain *c-myc* signals but the one at the top of *f* does not react with the ankyrin probe. Weak hybridization signals on other chromo-

somes (*a*, *b*, *e* and *f*) are due to background hybridization to Alu DNA sequences. *g*, Maps of human chromosome 8 indicating distribution of fractional length positions of fluorescent ankyrin signals (vertical bar). These correspond to an approximate location of the ankyrin gene at band 8p11.2. METHODS. Fluorescence *in situ* hybridization reactions were performed as described²⁷. The probes were biotin labelled and detected by incubation with FITC-conjugated avidin. DNA was counterstained with propidium iodide. The ankyrin probe was a 17-kilobase clone (BT 107) from a human genomic library³⁴, subcloned into plasmid pGEM (Promega, Madison, Wisconsin). It contains exons encoding a portion of the ankyrin C terminus. The range of fractional length values depicted in *g* were obtained by measurements on 20 extended (prometaphase) chromosomes.

spectrin (79%) and protein 4.2 (74%). This agrees with evidence that spectrin²⁸ and protein 4.2 (ref. 29) bind to ankyrin. In fact the deficiencies may even be greater, because the content of each protein is normalized by comparison to AE1, and HS red cells lose 15–20% of their membrane surface³⁰, including AE1 (ref. 2).

In conclusion, we find that the gene for human erythrocyte ankyrin is located at chromosome 8p11.2, probably at *SPH2*. In patients with interstitial deletions or translocations of chromosome 8 involving *SPH2*, ankyrin deficiency and associated deficiencies of spectrin and protein 4.2 are probably responsible for spherocytosis and haemolysis. Such gross chromosome defects are presumably rare in HS, but there is evidence that ankyrin defects may not be. First, two patients have been identified with an atypical form of HS and roughly half normal levels of spectrin and ankyrin³¹. Second, SDS-PAGE analysis of red cell membranes from more typical dominant HS patients, reveal mild deficiencies (15–25%) of both spectrin and ankyrin in 5 out of 11 patients (O.S. & S.E.L., unpublished observations) and 9 out of 12 patients (J. Palek, personal communication). Finally, there is evidence that functional ankyrin defects also exist. An *NcoI* ankyrin polymorphism is tightly linked to the locus for dominant HS in a large family with mild spectrin deficiency but no ankyrin deficit (F. Costa *et al.*, unpublished observations). Presumably the ankyrin mutation affects spectrin binding, although this has not been tested directly. □

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Transposition of an antibiotic resistance element in mycobacteria

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BACTERIAL resistance to antibiotics is often plasmid-mediated and the associated resistance genes encoded by transposable elements. Mycobacteria, including the human pathogens *Mycobacterium tuberculosis* and *M. leprae*, are resistant to many antibiotics, and their cell-surface structure is believed to be largely responsible for the wide range of resistance phenotypes. Antibiotic-resistance plasmids have so far not been implicated in resistance of mycobacteria to antibiotics. Nevertheless, antibiotic-modifying activities such as aminoglycoside acetyltransferases¹ and phosphotransferases¹ have been detected in fast-growing species^{2,3}. β -lactamases have also been found in most fast- and slow-growing mycobacteria. To date no mycobacterial antibiotic-resistance genes have been isolated and characterized. We now report the isolation, cloning and sequencing of a genetic region responsible for resistance to sulphonamides in *M. fortuitum*. This region also contains an open reading frame homologous to one present in Tn1696⁴ (member of the Tn21 family) which encodes a site-specific integrase^{5,6}. The mycobacterial resistance element is flanked by repeated sequences of 880 base pairs similar to the insertion elements of the IS6 family found in Gram⁺ and Gram⁻ bacteria. The insertion element is shown to transpose to different sites in the chromosome of a related fast-growing species, *M. smegmatis*. The characterization of this element should permit transposon

mutagenesis in the analysis of mycobacterial virulence and related problems.

We have analysed different fast growing mycobacterial strains resistant to antibiotics of clinical importance. Chromosomal DNAs were digested with *PstI* and hybridized with probes derived from a variety of antibiotic-resistance genes and transposons. DNA from *M. fortuitum* FC1, a natural isolate, hybridized to a probe that included the gentamicin-resistance gene from Tn1696⁴. We determined the nucleotide sequence of the cross-hybridizing region (Fig. 1). Similarities between the Tn1696 probe and the cloned fragment are located 5' to the gentamicin-resistance gene. In Tn1696 this region contains an open reading frame (orf2) encoding an integrase known to be involved in *recA*-independent site-specific recombination^{5,6}. The mycobacterial sequence contains an open reading frame (orf2M) similar to this site-specific integrase. An open reading frame (*sul3*), homologous to the sulphonamide-resistance gene *sul1*, also present in Tn1696 and in other members of the Tn21 family, was found downstream of orf2M. Both *M. fortuitum* FC1, and *Escherichia coli* transformed with a plasmid (pIPC1) that contains the region of cross-hybridization (legend Fig. 1), were highly resistant to sulphonamides, demonstrating that *sul3* is expressed in both *E. coli* and mycobacteria.

To define the *sul3*-containing region more precisely, we cloned and sequenced its flanking regions in the recombinant plasmid pIPC10 (legend Fig. 1). Immediately upstream of orf2M was an 880 base pair (bp) sequence, also found repeated downstream from *sul3*. Similarities were observed between these repeated sequences and the insertion sequence IS15 Δ , which belongs to the IS6 family^{7–9}. The mycobacterial insertion sequence (termed IS6100), contains 14-bp inverted repeats with only one nucleotide that is different from those of IS15 Δ . A comparison of the putative IS6100 transposase with other IS6-like sequences shows that IS6100 is closely related to elements isolated from Gram⁻ and Gram⁺ bacteria (Fig. 2). Codon usage of the putative transposase of IS6100 was similar to that found in the gene encoding the main antigen protein of *M. tuberculosis* (relative molecular mass of 65,000 (*M_r* 65K))¹⁰ suggesting that

FIG. 1 Complete nucleotide sequence of Tn610. Deduced amino-acid sequences of the different open reading frames are indicated above and below the nucleotide sequence depending on the direction of transcription. Sequence of the *Sau*3A insert from plasmid pIPC1 is indicated by asterisks. Flanking regions of this fragment were sequenced from the insert of pIPC10. IS6100 inverted repeats are boxed. Nucleotide sequence is numbered from the beginning of the first insertion sequence (IS6100L). The amino-acid sequence of the putative transposase is indicated above the second insertion sequence (IS6100R). N-terminal amino-acid sequences of *orf*2M and *sul*3 that differ from the corresponding sequences of Tn21 are indicated in italics. The different SD, -10 and -35 consensus sequences are underlined.

METHODS. Total chromosomal DNA from *M. fortuitum* FC1 was prepared as follows: a 100-ml culture in 7H9 Middlebrook medium was treated for protoplast formation, centrifuged, and resuspended in 5 ml of 25% sucrose, 50 mM Tris buffer, pH8.0, 50 mM EDTA, 500 μ g ml⁻¹ lysozyme and incubated for 1 h at 37 °C. 100 mM Tris buffer, pH8.0, 400 μ g ml⁻¹ proteinase K, 1.0% SDS (5 ml total) was added and the mixture was incubated (55 °C 2 h). DNA was then extracted with phenol-chloroform and ethanol precipitated. Standard procedures were used for DNA cleavage, ligation and transformation¹⁶. Construction of plasmid pIPC1: After partial digestion of total *M. fortuitum* FC1 DNA with *Sau*3A, fragments of 1–5 Kilobases (kb) were inserted in the *Bam*HI site of the pUC18 cloning vector. The 3-kb *Bam*HI fragment from the plasmid pSK6 (ref. 17) labelled with [³²P] dCTP by the random primer method (Amersham), was used as a probe to screen the library by colony hybridization and a 1.5-kb fragment inserted in pUC18 was selected (pIPC1). Filters were hybridized at high stringency: 50% formamide, 5×SSPE, 2×Denhardt's solution at 42 °C and washed in 0.1×SSC, 0.1% SDS at 65 °C. Construction of pIPC10: Southern blot analysis revealed a 7.5 kb *Eco*RI fragment that cross-hybridized with pIPC1. Total *M. fortuitum* FC1 DNA was digested with *Eco*RI, and fragments of 5–10 kb were randomly inserted into the *Eco*RI site of pUC18. Transformants were selected for resistance to sulphamamide. The recombinant plasmid, pIPC10, was isolated, and regions of the insert adjacent to *sul*3 were sequenced. Plasmid pIPC17 was constructed by replacing the internal *Eco*RV fragment of *sul*3 in pIPC10, with a *Hin*clI fragment containing the kanamycin resistance gene from Tn903. Nucleotide sequences were determined by the dideoxy chain termination method¹⁸ using the Sequenase system (USB) or the *Taq*-track system (Promega-Biotec). For sequencing, restriction fragments were subcloned in M13mp18 and M13mp19 vectors.

-188	TCGCCATTGT	GAACATGCAA	ATATCCGATT	TGCGGGCTGT	CGAGAGATCC	GTCAACAGCC	ACCAGGTCGG
-118	CCTTTGCCCG	TGATAGCGTC	GCCCCAGGCA	GACCGTTTAT	CCATCTGAGG	GCACGGAATA	TGCCCAACAA
-48	TTCCATGGGT	GATTTCGTCA	GCCAGTTGCG	CCCGCGATTA	CATCGCGG	<u>GGCTCTGTGGA</u>	AGGATTGGCG
23	GCAGTCAGAG	GTAGGCTGTC	GCTCTGCGCC	GATCAGGCGG	GTGTTGCGAA	ATGGTGGTTG	ACGATGCCCA
93	TGGCCTCCGT	CAGCGCCGAG	GGCCCAATGC	CAAAAGCTCT	CTCCACAAGG	CGCACCTCGC	CCCTGATGCG
163	GGGCTCGAGG	CACCAAGGGC	GAGCCTGTCC	TTTGCCGAGG	GCTCGCATGA	CTTCAATCC	CTTGATCGTC
233	GCATAGGCCG	TGGGGATCGA	TTTAAACCG	CGCACCGGCT	TGATCAGTAT	CTTGAGCTTT	CCGTGATCGG
303	CCTCGATCAC	GTTATTGAGA	TACTTCACCT	GCCGTTGGGC	CGTCTCCCGG	TCCAGGTTTC	CTTCCGGCTT
373	CAATTCCGSG	ATCGCTGCAC	CATAGCTCGG	CGCTTTGTCT	GTATTGAGCG	TGGCAGGCTT	TGCCAGCTGC
443	TTCAGGCCTC	GCAGGCGCTT	GCCAGGAAAC	CGCTTCGCTG	CCTTGGCGCT	CGGGGTCGGC	GACAGGTAGA
513	AATCGATCGT	GTGCCCCCGC	TTGTGCACTG	CCCGGTACAG	GTAGGTCAC	TTGCCCCGCA	CCITGACGTA
583	GGTTTCATCC	AGGCGCCAGC	TCGGATCAAA	GCCACGCCGC	CAGAACCAGC	GCAGCCGCTT	CTCCATCTCC
653	GGGGCGTAGC	ACTGGACCCA	CGCATAGATC	GTCTGATGGT	CGACCGAAAT	GCCGCGTTCC	GCCAGCATTT
723	CCTCAAGGTC	GCATAGCTGT	ATCGGATAGC	GACAATACCA	CGGCACCGCC	CACAGGATCA	CATCACCGTG
793	GAAATGGCGC	CACCTGAAAT	CCGTATCATG	TCCGTCCGTC	CAATCTCCGC	CAAGCATGCT	CA AGC TTC
							Ala Glu
861	ACG ATT	<u>TTT GGA ACA GAG CGG</u>	AAT GTC	GTA ACC	GCT GCG	GAG CAA	GGC CGT
	Arg Asn	Lys Cys Cys Leu Arg	Ile Asp	Tyr Gly	Ser Arg	Leu Leu	Ala Thr
921	GTG GCG	GAG GGT	GTG CGG	TGT GGC	GGG CTT	CGT GAT	GCC TGC
	His Arg	Leu Thr	His Pro	Thr Ala	Pro Lys	Thr Ile	Gly Ala
981	GAA GGC	GCG CTG	AAA GGT	CTG GTC	ATA CAT	GTG ATG	GCG ACG
	Phe Ala	Arg Gln	Phe Thr	Gln Asp	Tyr Met	His His	Arg Arg
1041	ATC GGT	CGA ATG	CGT GTG	CTG CGC	AAA AAC	CCA GAA	CCA CGG
	Asp Thr	Ser His	Thr His	Gln Ala	Phe Val	Trp Phe	Trp Pro
1101	CGG ATA	CTT CCG	CTC AAG	GGC GTC	GGG AAG	CGC AAC	GCC GCT
	Pro Tyr	Lys Arg	Glu Leu	Ala Asp	Pro Leu	Ala Val	Gly Ser
1161	CTT CAG	CCA CCA	TGC CCG	TGC ACG	CGA CAG	CTG CTC	GCG CAG
	Lys Leu	Trp Trp	Ala Arg	Ala Arg	Ser Leu	Gln Glu	Arg Leu
1221	GGG TAA	CAT CAA	GGC CCG	ATC CTT	GGA GCC	CTT GCC	CTC CCG
	Pro Leu	Met Leu	Ala Arg	Asp Lys	Ser Gly	Lys Gly	Glu Arg
1281	ATC GAA	ATC CAG	ATC CTT	GAC CCG	CAG TTG	CAA ACC	CTC ACT
	Asp Phe	Asp Leu	Asp Lys	Val Arg	Glu Leu	Gln Leu	Gly Ser
1341	ATA CAG	AAG CTG	GGC GAA	CAA ACG	ATG CTC	GCC TTC	CAG AAA
	Tyr Leu	Leu Gln	Ala Phe	Leu Arg	His Glu	Gly Glu	Leu Phe
1401	TTC ATC	CGG GGT	CAG CAC	CAC CGG	CAA GCG	CCG CGA	CGG CCG
	Glu Asp	Pro Thr	Leu Val	Val Pro	Leu Arg	Arg Ser	Pro Arg
1461	AAG CCA	GGG CAG	ATC CGT	GCA CAG	CAC CTT	GCC GTA	GAA GAA
	Leu Trp	Pro Leu	Asp Thr	Cys Leu	Val Lys	Gly Tyr	Phe Phe
1521	CTG ACG	ATG CGT	GGA GAC	CGA AAC	CTT GCG	CTC GTT	CGC CAG
	Gln Arg	His Thr	Ser Val	Ser Val	Lys Arg	Glu Asn	Ala Leu
1581	GAC TTC	GCT GCT	GCC CAA	GGT TGC	CGG GTG	ACG CAC	ACC GTG
	Val Glu	Ser Ser	Gly Leu	Thr Ala	Pro His	Arg Val	Gly His
1641	AAC CCA	<u>GTG GAC ATA</u>	AGC CTG	TTC GGT	TCG TAA	<u>GCT GTA</u>	ATG CAA
	Val Trp	His Val	Tyr Ala	Gln Glu	Thr Arg	Leu Ser	Tyr His
1701	ACG CAA	CTG GTC	CAG AAC	CTT GAC	CGA ACG	CAG CGG	TGG TAA
	Arg Leu	Gln Asp	Leu Val	Lys Val	Ser Arg	Leu Pro	Pro Leu
1761	CAT GGCTTGTTAT	GACTGTTTTT	TTGTACAGTC	TATGCCTCGG	GCATCCAAGC	AGCAAGCGCG	TTACGCCGTG
	Met	-----	<i>orf</i> 2M	-10	-35		
1834	GGTCGATGTT	TGATGTTATG	GACGACCAAC	G ATG	TTA CGC	AGC AGG	GTG ACG
	Leu Asn	Leu Thr	Glu Asp	Ser Phe	Phe Asp	Glu Ser	Arg Arg
1898	CTG AAT	CTC ACC	GAG GAC	TCC TTC	TTC GAT	GAG AGC	CGG CGG
	Val Thr	Ala Ala	Ile Glu	Met Leu	Arg Val	Gly Ser	Asp Val
1958	GTC ACC	GCG GCG	ATC GAA	ATG CTG	CGA GTC	GGA TCA	GAC GTC
	Ala Ser	His Pro	Asp Ala	Arg Pro	Val Ser	Pro Ala	Glu Ile
2018	GCC AGC	CAT CCG	GAC GCG	AGG CCT	GTA TCG	CCG GCC	GAT GAC
	Leu Leu	Asp Ala	Leu Ser	Asp Gln	Met His	Arg Val	Ser Ile
2078	CTC TTA	GAC GCC	CTG TCC	GAT CAG	ATG CAC	CGT GTT	TCA ATC
	GAC						

the insertion sequences are mycobacterial in origin. The compound transposon-like element consisting of *orf*2M, *sul*3 and the flanking IS6100 sequences, is termed Tn610. Two additional copies of IS6100 were detected in the chromosome of *M. fortuitum* FC1 by Southern blot analyses. But cross-hybridizing sequences were not found in other strains of *M. fortuitum*, or in other species, including *M. smegmatis* and *M. tuberculosis*.

The ability of Tn610 to transpose was tested in *M. smegmatis* mc²155 after electroporation with a non-replicating plasmid pIPC17 (legend to Fig. 1) in which *sul*3 is replaced by the

kanamycin-resistance gene of Tn903, which is expressed in mycobacteria¹¹. Kanamycin-resistant colonies (Km^r), that might correspond to chromosomal insertions of the transposon, were obtained at a frequency of <5 per 10⁶ μ g DNA. By contrast, 10⁴ transformants per μ g DNA were obtained by electroporation with the pAL8 *E. coli*-mycobacteria shuttle vector^{11,12}. No Km^r transformants were obtained when DNA of a pUC18 derivative containing the kanamycin-resistance gene from Tn903 was used instead of pIPC17.

The mechanism described for the transposition of IS6-family


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2138 Thr Gln Arg Tyr Ala Leu Lys Arg Gly Val Gly Tyr Leu Asn Asp Ile Gln Gly Phe Pro
ACC CAG CGC TAT GCG CTC AAG CGC GGC GTG GGC TAC CTG AAC GAT ATC CAA GGA TTT CCT

2198 Asp Pro Ala Leu Tyr Pro Asp Ile Ala Glu Ala Asp Cys Arg Leu Val Val Met His Ser
GAC CCT GCG CTC TAT CCC GAT ATT GGT GAG GCG GAC TGC AGG CTG GTG GTT ATG CAC TCA

2258 Ala Gln Arg Asp Gly Ile Ala Thr Arg Thr Gly His Leu Arg Pro Glu Asp Ala Leu Asp
GCG CAG CGG GAT GGC ATC GCC ACC CGC ACC GGT CAC CTT CGA CCC GAA GAC GCG CTC GAC

2318 Glu Ile Val Arg Phe Phe Glu Ala Arg Val Ser Ala Leu Arg Arg Ser Gly Val Ala Ala
GAG ATT GTG CGG TTC TTC GAG GCG CGG GTT TCC GCC TTG CGA CGG AGC GGG GTC GCT GCC

2378 Asp Arg Leu Ile Leu Asp Pro Gly Met Gly Phe Phe Leu Ser Pro Ala Pro Glu Thr Ser
GAC CGG CTC ATC CTC GAT CCG GGG ATG GGA TTT TTC TTG AGC CCC GCA CCG GAA ACA TCG

2438 Leu His Val Leu Ser Asn Leu Gln Lys Leu Lys Ser Ala Leu Gly Leu Pro Leu Leu Val
CTG CAC GTG CTG TCG AAC CTT CAA AAG CTG AAG TCG GCG TTG GGG CTT CCG CTA TTG GTC

2498 Ser Val Ser Arg Lys Ser Phe Leu Gly Ala Thr Val Gly Leu Pro Val Lys Asp Leu Gly
TCG GTG TCG CGG AAA TCC TTC TTG GGC GCC ACC GTT GGC CTT CCT GTA AAG GAT CTG GGT

2558 Pro Ala Ser Leu Ala Ala Glu Leu His Ala Ile Gly Asn Gly Ala Asp Tyr Val Arg Thr
CCA GCG AGC CTT GCG GCG GAA CTT CAC GCG ATC GGC AAT GGC GCT GAC TAC GTC CGC ACC

2618 His Ala Pro Gly Asp Leu Arg Ser Ala Ile Thr Phe Ser Glu Thr Leu Ala Lys Phe Arg
CAC GCG CCT GGA GAT CTG CGA AGC GCA ATC ACC TTC TCG GAA ACC CTC GCG AAA TTT CGC

2678 Ser Arg Asp Ala Arg Asp Arg Gly Leu Asp His Ala
AGT CGC GAC GCC AGA GAC CGA GGG TTA GAT CAT GCC TAGCATTAC CTTCCGCCCG CCCGCTAGCG
2744 GACCCTGGTC AGGTTCGCG AGGTGGGCG CAGACATGCT GGGCTCGTCA GGATCAAACT GCACTATGAG
2814 CGCGCGGTTC ATACCGCGCC AGGGGAGCGA ATGGACAGCG AGGAGCCTCC GAACGTTCCG GTCGCTGCT
2884 CGGGTGATAT CGACGAGGTT GTGCGGCTGA TGCACGACGC TCGCGCGTGG ATGTCGCGCA AGGGAACGCC
2954 CGCCTGGGAC GTCGCGCGGA TCGACCGGAC ATTGCGCGAG ACCTTCGTCC TGAGATCCGA GTCCTAGTC
3024 GCGAGTTGCA GCGACGGCAT CGTCGGCTGT TGCACCTTGT CGGCCGAGGA TCCGAGTTC TGCCCCGCGC

tnp ----> Met Thr Asp Phe
3094 TCGTTCGAA AATCGTGAA GCTTGAGCAT GCTTGGCGGA GATTGGACG ACGGAACG ATG ACG GAT TTC
-35 -10 SD
3164 Lys Trp Arg His Phe Gln Gly Asp Val Ile Leu Trp Ala Val Arg Trp Tyr Cys Arg Tyr
AAG TGG CGC CAT TTC CAG GGT GAT GTG ATC CTG TGG GCG GTG GCG TGG TAT TGT CGC TAT

3224 Pro Ile Ser Tyr Arg Asp Leu Glu Glu Met Leu Ala Glu Arg Gly Ile Ser Val Asp His
CCG ATC AGC TAT CGC GAC CTT GAG GAA ATG CTG GCG GAA CGC GGC ATT TCG GTC GAC CAT

3284 Thr Thr Ile Tyr Arg Trp Val Gln Cys Tyr Ala Pro Glu Met Glu Lys Arg Leu Arg Trp
ACG ACG ATC TAT CGC TGG GTC CAG TGC TAC GCC CCG GAG ATG GAG AAG CGG CTG CGC TGG

3344 Phe Trp Arg Arg Gly Phe Asp Pro Ser Trp Arg Leu Asp Glu Thr Tyr Val Lys Val Arg
TTC TGG CGG CGT GGC TTT GAT CCG AGC TGG CGC CTG GAT GAA ACC TAC GTC AAG GTG CGG

3404 Gly Lys Trp Thr Tyr Leu Tyr Arg Ala Val Asp Lys Arg Gly Asp Thr Ile Asp Phe Tyr
GGC AAG TGG ACC TAC CTG TAC CGG GCA GTC GAC AAG CGG GGC GAC ACG ATC GAT TTC TAC

3464 Leu Ser Pro Thr Arg Ser Ala Lys Ala Ala Lys Arg Phe Leu Gly Lys Ala Leu Arg Gly
CTG TCG CCG ACC CGC AGC GCC AAG GCA GCG AAG CGG TTC CTG GGC AAG GCC CTG CGA GGC

3524 Leu Lys His Trp Glu Lys Pro Ala Thr Leu Asn Thr Asp Lys Ala Pro Ser Tyr Gly Ala
CTG AAG CAC TGG GAA AAG CCT GCC ACG CTC AAT ACC GAC AAA GCG CCG AGC TAT GGT GCA

3584 Ala Ile Thr Glu Leu Lys Arg Glu Gly Lys Leu Asp Arg Glu Thr Ala His Arg Gln Val
GCG ATC ACC GAA TTG AAG CGC GAA GGA AAG CTG GAC CGG GAG ACG GCC CAC CGG CAG GTG

3644 Lys Tyr Leu Asn Asn Val Ile Glu Ala Asp His Gly Lys Leu Lys Ile Leu Ile Lys Pro
AAG TAT CTC AAT AAC GTG ATC GAG GCC GAT CAC GGA AAG CTC AAG ATA CTG ATC AAG CCG

3704 Val Arg Gly Phe Lys Ser Ile Pro Thr Ala Tyr Ala Thr Ile Lys Gly Phe Glu Val Met
GTG CGC GGT TTC AAA TCG ATC CCC ACG GCC TAT GCC ACG ATC AAG GGA TTC GAA GTC ATG

3764 Arg Ala Leu Arg Lys Gly Gln Ala Arg Pro Trp Cys Leu Gln Pro Gly Ile Arg Gly Glu
CGA GCC CTG CGC AAA GGA CAG GCT CGC CCC TGG TGC CTG CAG CCC GGC ATC AGG GGC GAG

3824 Val Arg Leu Val Glu Arg Ala Phe Gly Ile Gly Pro Ser Ala Leu Thr Glu Ala Met Gly
GTG CGC CTT GTG GAG AGA GCT TTT GGC ATT GGG CCC TCG GCG CTG ACG GAG GCC ATG GGC

Met Leu Asn His His Phe Ala Ala Ala Ala
3884 ATG CTC AAC CAC CAT TTC GCA GCA GCC GCC TGATCGGCGC AGAGCGACAG CCTACCTCTGA
3944 CTGCGGCCAA TCATGCAAC AGAGCG GTCA GCATGACCAA CAGCCCTCGT GCGCGAATCG GCTTCCCTCT
4014 CCGGGCGCGT GTCTGGTGGG CGTCGGACAC CTGGCCCATC ACCGCGCGG CAGGAGGCTG

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elements in Gram⁻ bacteria, requires the formation of co-integrates as the end-products of transposition, with the integration of the donor vector plus one extra copy of the insertion sequence⁹. Km^r transformants were examined by Southern hybridization using an IS6100 internal fragment as a probe. As expected, in nine out of eighteen transformants (six are shown in Fig. 3) a novel IS6100-specific fragment was detected, in addition to both original plasmid bands. This extra fragment was different for each transformant tested. Hybridization of these nine clones with pIPC17 confirmed in each case that the

entire plasmid sequence was integrated into the chromosome (Fig. 3). Hybridization with probes containing sequences flanking the IS6100 repeats confirmed that the novel IS6100 bands contained one new and one old junction (Fig. 4). These results are consistent with transposition occurring at different sites in the chromosome by a replicative transposition of one of the IS6100 elements of Tn610. In the nine other transformants only two copies of IS6100 were detected, indicating a different mechanism of recombination, which we are investigating.

IS6100	PTGFRHDFQVLLMAVAVKVPSTYRDIPEMAERGISVQHTTYRWVQVAPEMEKRLRMFW	(66)
IS240 B	MEKE-RLPKNKHQADLLNFWVLLNLSFDIDVEMERKGLSHTTTAFVWQVGPEDNRIRKHL	(69)
IS26	HSPTFGRHFDYLLMAVAVKVPSTYRDIPEMAERGISVQHTTYRWVQVAPEMEKRLRMFW	(66)
ISS1	HSPTFGRHFDYLLMAVAVKVPSTYRDIPEMAERGISVQHTTYRWVQVAPEMEKRLRMFW	(66)
IS431	HSPTFGRHFDYLLMAVAVKVPSTYRDIPEMAERGISVQHTTYRWVQVAPEMEKRLRMFW	(66)
IS6100	RRGDFPS-MRLDETIVKVRGKVIYLYRAVDKCHTIDFYLSPTSAKAKKRFICQKRLGLKHWKPATIN	(135)
IS240 B	KRTNDS--NRVEETIKIKGNMILYRAVDKCHTIDFYLSPTSAKAKKRFICQKRLGLKHWKPATIN	(136)
IS26	RNPSEILCPMDDETIVKVRGKVIYLYRAVDKCHTIDFYLSPTSAKAKKRFICQKRLGLKHWKPATIN	(136)
ISS1	RDSFYS--MMDDETIVKVRGKVIYLYRAVDKCHTIDFYLSPTSAKAKKRFICQKRLGLKHWKPATIN	(131)
IS431	KRAYTK-MRLDETIVKVRGKVIYLYRAVDKCHTIDFYLSPTSAKAKKRFICQKRLGLKHWKPATIN	(132)
IS6100	TDKAPSYGAAITELKREGKIDETANRQVYKYNMVEQDRHILKGRIRMLGLKSMCTAYKAFETAME	(205)
IS240 B	VDEKAVIVAIRELKHKISYQMLHVKYNNMIEQDRHILKGRIRMLGLKSMCTAYKAFETAME	(206)
IS26	TDKARAGRAIALKREGKIDETANRQVYKYNMVEQDRHILKGRIRMLGLKSMCTAYKAFETAME	(206)
ISS1	TDKAPSYGAAITELKREGKIDETANRQVYKYNMVEQDRHILKGRIRMLGLKSMCTAYKAFETAME	(197)
IS431	TDKAPSYGAAITELKREGKIDETANRQVYKYNMVEQDRHILKGRIRMLGLKSMCTAYKAFETAME	(197)
IS6100	ALRKGQRPWCLPGIRGEVRLVERAFGLTPEALTRAMGMNHFFAANA	(254)
IS240 B	MVKKGQLLRAQ-----SAGNQRGTHQ-LFGLTN	(235)
IS26	ALRKGQSAF-----NYGDFLGMR--VSRVFEM	(234)
ISS1	GLTTPHNGTL-----FGFVSTELKV-LMGILN	(226)
IS431	TEVAPHSLSQT-----KGFSCHESSIMAS	(274)

FIG. 2 Comparison of the deduced amino-acid sequence of the putative transposase of IS6100 with those of the IS6 family. The entire sequences are shown; IS240 was isolated from *Bacillus thuringiensis*¹⁹, IS26 from *Proteus vulgaris*²⁰, similar to IS15Δ from *Salmonella panama*⁷, ISS1 from *Streptococcus lactis*²¹ and IS431 from *Staphylococcus aureus*²². They are aligned using the computer program of W. Saurin and P. Marlière²³. Gaps are represented by spaces. Amino-acids identical or functionally related in at least three sequences are indicated by shaped boxes. (Adapted from Delecluse *et al.*¹⁹.)

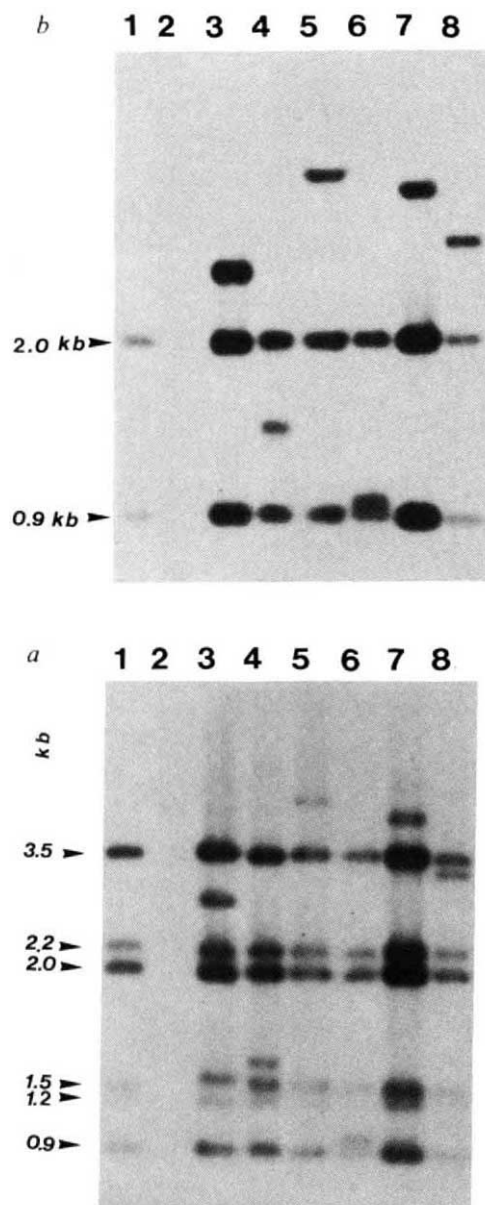


FIG. 3 Southern blot hybridization of the product of transposition of IS6100 in *M. smegmatis*. Genomic DNA (5 µg) was digested with PstI (10 U) for 3 h. After electrophoresis on a 0.7% agarose gel, the DNA was transferred to a Hybond-N membrane filter (Amersham). DNA blots were hybridized at high stringency. Experimental conditions were as described in the Fig. 1 legend. The order in the three experiments is the same: Lane 1, pIPC17 PstI; lane 2, DNA from *M. smegmatis* mc²¹⁵⁵; lanes 3–8, independent Km^r clones of *M. smegmatis* mc²¹⁵⁵ transformed with pIPC17. *a*, An internal PstI–HindIII fragment of IS6100 (nucleotides 166–854, Fig. 1) was used as a probe. A novel fragment, different in size for each clone and corresponding to one end of the site of insertion into the chromosome, was identified. Hybridizations with a fragment of IS6100 (nucleotides 1–163) corresponding to the end of the insertion sequence, and outside the HindIII–PstI fragment, identified new and different fragments for each clone, corresponding to the other end of the site of insertion (data now shown). *b*, A mixture of the PstI-generated fragments of pIPC17 was used as probe (random primer method). All of the pIPC17 PstI fragments were detected in each clone, demonstrating that the plasmid was indeed integrated and arguing against homologous recombination into the chromosome or the involvement of an integrase.

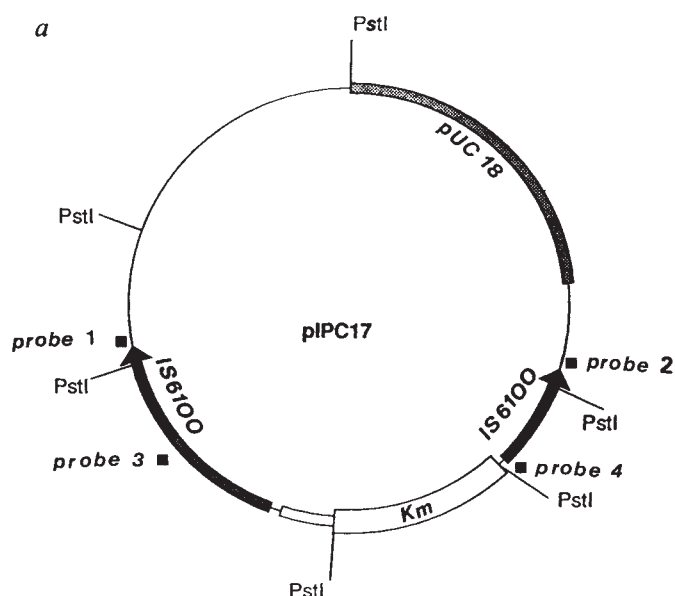


FIG. 4 Southern blot hybridization analysis with probes containing IS6100 flanking sequences. The PstI-digested DNA fragments were hybridized with probes corresponding to sequences flanking the ends of the insertion sequence. *a*, Location of the different probes. Probe 1, a fragment from nucleotides –98–0, external side of IS6100L. Probe 2, fragment from nucleotides 3,970–4,055, external side of IS6100R; probe 3, fragment from nucleotides 881–1,508, internal side of IS6100L; probe 4, fragment from nucleotides 3,007–3,090, internal side of IS6100R (probes generated by *in vitro* DNA amplification of these fragments by the polymerase chain reaction, using as primers 22-residue oligonucleotides (22-mers) 5' and 3' to the sequence and adding [³²P] dCTP to the mixture of dNTPs). *b*, Lane 1, pIPC17 PstI; lane 2, DNA from *M. smegmatis* mc²¹⁵⁵; lanes 3–8, independent Km^r clones of *M. smegmatis* mc²¹⁵⁵ transformed with pIPC17. Experimental conditions were as described in Fig. 3 legend. Hybridization with probes 1–4 revealed the corresponding region in all of the clones and no extra fragments were detected. No hybridization was detected with the fragments previously identified as ends of the site of insertion of IS6100 (Fig. 3*a*). This confirmed the breaking of the link to the original flanking DNA, and insertion into a new DNA sequence.

Members of the Tn21 family all possess an integrase (orf2) and a sulphonamide-resistance determinant (*sul1*), with a variety of different antibiotic-resistance genes inserted by recombination at a specific site between these two genetic elements. The Tn21 family probably evolved by insertion of different antibiotic-resistance genes by site-specific recombination into an evolutionarily older and simpler structure containing these two elements⁶. Sulphonamides have been used since 1935 and were employed in the treatment of tuberculosis in the early 1950s. It is therefore not surprising that sulphonamide resistance is associated with a transposable element in mycobacteria. That Tn610 is structurally related to the Tn21 family¹³, possesses an integrase (orf2M) and a sulphonamide-resistance gene (*sul3*) but without additional antibiotic-resistance genes, indicates that Tn610 may be derived from an ancestral transposon containing only the gene responsible for resistance to sulphonamide.

Genetic analysis in mycobacteria has been restricted by the lack of systems for generating mutants^{11,12,14,15}. Transposon mutagenesis with IS6100 will facilitate the analysis of virulence in pathogenic strains such as *M. tuberculosis*, and allow the preparation of avirulent strains useful in vaccine development. This system could also be used to exploit the strong immunostimulant properties of the live vaccine *M. bovis* BCG. Stable transposon-mediated insertion of foreign epitopes should allow the construction of polyvalent BCG recombinant vaccines. □

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Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*

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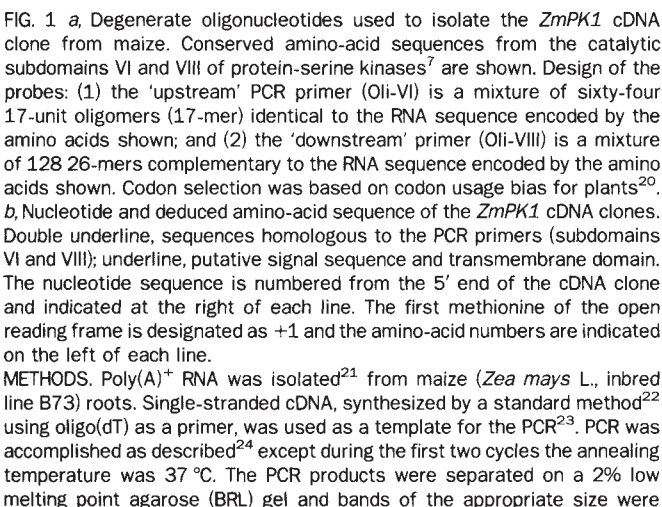
THE protein kinase family of enzymes mediates the responses of eukaryotic cells to both inter- and intracellular signals^{1,2}. These enzymes are either serine/threonine-specific or tyrosine-specific. Many of the latter are transmembrane receptors and are important in transduction of extracellular signals across the plasma membrane³, whereas few examples of receptor serine kinases have been reported⁴. We have now identified a complementary DNA clone from *Zea mays* (L.) encoding a putative serine/threonine-specific protein kinase structurally related to the receptor tyrosine kinases. This structural similarity is evidence for a previously undescribed class of transmembrane receptor in higher plants likely to be involved in signal reception and transduction. Furthermore, the catalytic domain of this protein kinase is linked through a transmembrane domain to an extracellular domain similar to that of glycoproteins encoded in the self-incompatibility locus of *Brassica* which are involved in the self-recognition system between pollen and stigma^{5,6}.

The catalytic domains of protein kinases consist of key amino-acid residues nested in blocks of conserved protein sequence⁷. Several groups have taken advantage of this conservation and used oligonucleotides as hybridization probes^{8–10} or polymerase chain reaction (PCR) primers^{11,12} to clone protein kinase genes and cDNAs. We used the PCR technique to identify and isolate in maize cDNAs that encode protein kinases. Mixed oligonucleotides (Fig. 1a) encoding amino-acid sequences conserved in the serine/threonine-specific protein kinases⁷ were synthesized and used as primers to amplify cDNAs, which were then cloned and sequenced. Clones containing both primers and flanking a sequence encoding an in-frame Asp-Phe-Gly, which

is highly conserved in the protein kinases⁷, were isolated and used as hybridization probes to isolate clones from a maize root cDNA library.

Nucleotide sequence analysis (Fig. 1b) of one of the maize putative protein kinase-encoding cDNA clones (*ZmPK1*) revealed an open reading frame encoding a protein of 817 amino acids with a relative molecular mass of 91,088. This sequence represents a nearly complete transcript; northern blots indicate that the *ZmPK1* messenger RNA is ~2,700 nucleotides (see Fig. 3a). The first 28 amino acids constitute a hydrophobic N-terminal signal peptide¹³; hydropathic analysis¹⁴ predicts a transmembrane domain from residues 473 to 498 followed by a Lys-Arg stop transfer sequence¹⁵. The predicted extracellular domain contains 444 amino-acid residues, 6 potential N-linked glycosylation sites and 13 cysteine residues which are clustered near the transmembrane domain. The amino-acid sequence in the catalytic subdomains VI and VIII of eukaryotic protein kinases are specific to either the serine/threonine or the tyrosine kinases⁷. The predicted *ZmPK1* amino-acid sequence is typical of the serine/threonine kinases: subdomains VI (DVKPEN; single-letter amino-acid code) and VIII (GTLGYIAPE) (Fig. 1b) are similar to the consensus sequences found in known serine/threonine kinases (VI, DLKPEN; VIII, GTPXYIAPE) rather than the sequences found in the tyrosine kinases (VI, DLAARN; VIII, FPIKWMape).

The putative catalytic domain of the *ZmPK1* protein is related to the *raf* family of serine/threonine protein kinases (Fig. 2), cytoplasmic enzymes not associated with membranes. There is 27% identity and 55% conservation between the deduced amino-acid sequence of *ZmPK1* and the *raf-1* gene product sequence from humans¹⁶. There is also considerable similarity to the epidermal growth factor receptor (EGFR) family of tyrosine kinases (Fig. 2): 26% identity with human EGFR¹⁷ and 55% conservation. We were not surprised by the similarity of the *ZmPK1* amino-acid sequence to both *Raf* and the EGFR because phylogenetic analysis of the protein kinases⁷ indicates that the tyrosine-specific kinases have the least divergence from the *raf* family of protein-serine/threonine kinases. In addition to the similarity between amino-acid sequences in the *ZmPK1* protein and EGF receptors, these two molecules have several other structural similarities, including a cytoplasmic domain encoding a protein kinase, a single transmembrane domain and an



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2. Comparison of the deduced amino-acid sequence with the amino-acid sequences of the entire *s13* allele of *B. oleracea* S-locus glycoprotein (G-S13)¹⁸, the amino-acids 355–619 of the human *raf-1* gene product (raf-1)¹⁶ and the amino acids 705–981 of the human epidermal growth factor receptor (hEGFR)¹⁷. Single-letter amino-acid code is used. The positions of conserved amino acids (structurally similar R groups) are enclosed; similar letters, positions where there is identity. For the purposes of this figure, structurally similar amino acids are: nonpolar R groups (M, L, I, V, C); aromatic or ring-containing R groups (F, W, Y, H); small R groups with neutral polarity (A, G, S, T, P); acidic and uncharged polar R groups (D, N, Q); and basic polar R groups (R, K, H). Gaps introduced for optimal alignment are shown by dots. The transmembrane domain is underlined.

To determine if *ZmPK1* belongs to a family of related genes in maize, we performed a Southern blot of genomic DNA with the cDNA clone (Fig. 3b). Under high stringency hybridization conditions we observed four to five bands. Low stringency hybridizations (melting temperature -42°C) do not reveal any additional hybridizing bands (data not shown).

We propose that the protein encoded by the *ZmPK1* cDNA clone is one of a previously undescribed class of plant cell receptor. By analogy to the receptor tyrosine kinases in vertebrates, we postulate that a ligand interacts with the extracellular



METHODS. Poly(A)⁺ RNA was isolated as described in Fig. 1, electrophoresed on 6% formaldehyde/1% agarose gels, and transferred to Nylon membranes. Southern blots were prepared by standard procedures²². Filters were pre-hybridized and hybridized in 50% formamide, 100 µg ml⁻¹ sonicated salmon testes DNA, 100 µg ml⁻¹ yeast RNA, 5×Denhardt's, 50 mM sodium phosphate (pH 6.5), 5×SSC and 0.2% SDS at 42 °C for 12–16 h. Filters were hybridized with ~1×10⁶ c.p.m. per ml of the largest *ZmPK1* cDNA clone (2,700 bp) and washed in 2×SSC, 0.2% SDS at room temperature, then in 0.2% SSC, 0.2% SDS at 65 °C, and then autoradiographed with an intensifying screen. Exposure times: a, 24 h; b, 72 h.

domain of this protein and triggers the activation of the protein kinase moiety residing on the cytoplasmic side of the plasma membrane. The protein kinase then phosphorylates specific substrates, resulting in the production of second messengers, amplification of the signal and, ultimately, alteration or activation of cellular processes. This putative receptor is exceptional because it has sequence homology typical of the serine/threonine protein kinases. Most transmembrane protein kinases described so far are of the tyrosine class. The only other putative receptor serine kinase that we are aware of is the *daf-1* gene from the nematode *Caenorhabditis elegans*⁴. Although at this time there is no biochemical evidence that either the *daf-1* or the *ZmPK1* gene products are serine/threonine-specific protein kinases, the amino-acid sequence is strong evidence that these proteins represent a new type of signal-transducing molecule—receptor serine/threonine protein kinases. The identification of *ZmPK1* as a putative receptor protein kinase—to our knowledge the first transmembrane receptor identified in higher plants—provides a unique opportunity to gain fresh insights into signal transduction in higher plants. □

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CORRECTION

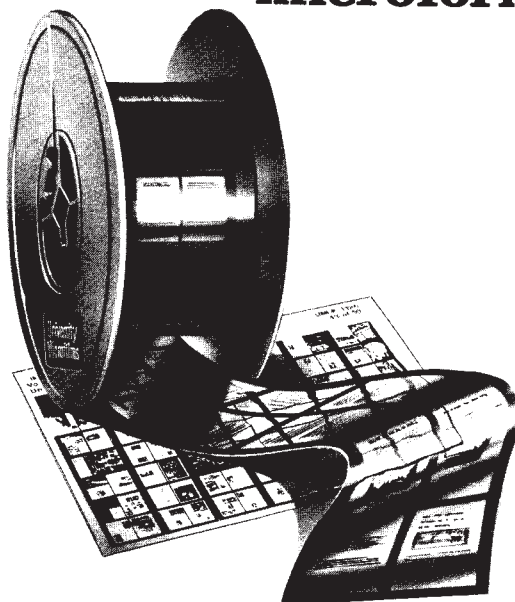
Organization of microtubules in dendrites and axons is determined by a short hydrophobic zipper in microtubule-associated proteins MAP2 and tau

Sally A. Lewis, Ivan E. Ivanov, Gwo-Hwa Lee & Nicholas J. Cowan

Nature **342**, 498–505 (1989).

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Electrospray ionization mass spectrometry of biomolecules

I. Jardine

The novel ionization technique of electrospray now allows the convenient mass spectrometric analysis of proteins and other biological molecules directly from liquids. The molecular weights of protein can be determined to better than 0.01 per cent mass accuracy.

In 1988, two new techniques for high mass analysis, which represented real breakthroughs in mass spectrometry, exploded onto the scene. They are matrix-assisted laser desorption ionization with time-of-flight mass analysis (LD-TOF), discovered by Karas and Hillenkamp of the University of Münster¹, and electrospray ionization of liquids with quadrupole mass analysis (ESI-quad), developed by Fenn and coworkers at Yale University².

Both of these techniques were quickly shown to possess the ability to measure accurately the molecular weight of proteins to over 100,000 Da at high sensitivity in the biochemically useful low-picomole-to-femtomole range (low nanogram quantities of protein), as well as nucleic acids to at least a few thousand daltons. The discussion in mass spectrometry circles quickly became one of not how to measure the molecular weight of proteins, but what were the pros and cons — relative mass range capabilities, sensitivities, mass accuracies, structural information obtainable, cost and ease of use — of LD-TOF versus ESI-quad.

Interestingly, these two techniques are quite different. Matrix-assisted laser desorption ionization generates predominantly singly-protonated protein ions that require the essentially unlimited mass range capabilities of a time-of-flight mass analyser. Commercial LD-TOF systems are not readily available, but should be in the near future. Electrospray ionization (ESI) on the other hand generates a series of multiply-protonated protein ions that usually fall in the mass/charge range of 500–2,000 Da, which is ideal for quadrupole mass analysers. Electrospray ionization-quad systems are commercially available because this requires only adaptation of electrospray to standard quadrupole analysers. Two reviews on the state-of-the-art in ESI are recommended^{3,4}.

ESI quadrupole hardware

A schematic diagram of an electrospray source coupled to a quadrupole mass spectrometer, which has been developed from research work at Yale⁵, is shown in Figure 1. The analyte in an appropriate solvent system such as methanol/water is introduced through a hollow stainless

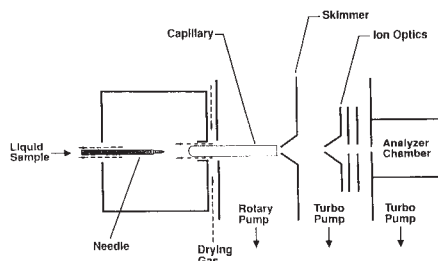


FIG. 1 Schematic diagram of an electrospray ion source attached to a quadrupole mass spectrometer. Ions created via electrospray at atmospheric pressure are transported through a glass capillary nozzle (0.5-mm i.d.), skimmer and ion optics into the mass analyser.

steel needle at a flow rate of 1–40 $\mu\text{l min}^{-1}$ to the ionization region located at the tip of the needle. Ionization is effected by applying a voltage of about $\pm 4,000$ V between the needle and the capillary (the needle is at ground potential and the voltage is positive/negative depending on whether positive or negative ions are to be analysed). The liquid eluting from the needle, under the influence of the high voltage, produces a fine spray of highly-charged droplets. The solvent in these droplets is evaporated rapidly by a high counter-current flow of warm (60 °C) nitrogen. The resulting dried and highly-charged analyte molecules are pulled through the capillary interface by the first pump, separated from neutral drying gas and solvent vapour at the skimmer and, subsequently, focused through the lensing system into the quadrupole mass spectrometer analyser. Sample solutions can be infused continuously into the needle using a low-pressure syringe pump, or samples can be introduced via loop injection or directly from separations systems such as high-performance liquid chromatography (HPLC) or high-performance capillary electrophoresis (HPCE).

ESI spectra

Initially, it was thought that the multiplicity of charge states generated by ESI of proteins (see Fig. 2a) would make interpretation of the spectra of unknown proteins difficult. In fact, data for pure unknowns are easy to interpret¹ and, in addition, the multiple ion series generates

multiple experimental data points, which improves the mass accuracy and precision of molecular weight measurement. Indeed, a modern calibrated high-performance quadrupole system can provide mass accuracies at a level of about 100 parts per million or better, or one part in 10,000. It is expected that the molecular weight determination of a 10,000-Da protein should be accurate to within ± 1 Da, and this is in fact the case¹. That is, in general, the determination of protein molecular weights by ESI with subsequent mass analysis on a quadrupole mass spectrometer will be accurate to within about 0.01 per cent. This is clearly a considerable improvement over SDS-PAGE molecular weight analysis.

Interpretation of electrospray data is made even easier with the implementation of novel software routines that automatically convert the relative abundance versus mass/charge (m/z) plots, as in

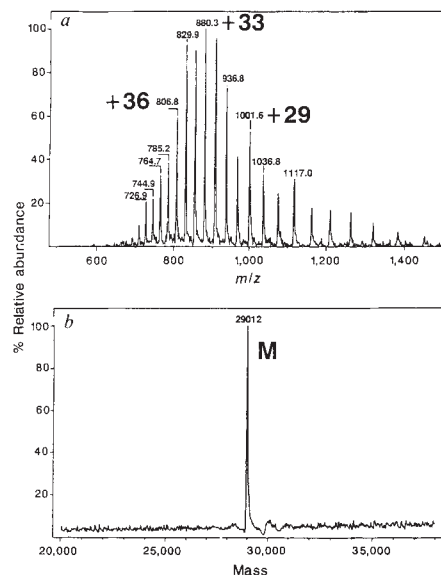


FIG. 2 a, Electrospray mass spectrum obtained from carbonic anhydrase (mol. wt. approximately 29,000 Da) infused into the electrospray ionization-quad system at a concentration of 3×10^{-6} M showing the multiply-protonated ion profile (not all ions have been labelled). b, Computer deconvolution of the relative abundance versus mass/charge profile data of a into a relative abundance versus mass plot from which the protein mass can be read directly.

Figure 2a, into relative abundance versus mass plots, as in Figure 2b. Such software is essential for the direct interpretation of complex overlapping multiply-charged envelopes of protein mixtures.

Although many proteins have been 'weighed' by ESI on a quadrupole system¹, to date, not all attempted analyses of proteins have been successful and the reasons for this are under investigation in several laboratories. In addition, it is important to note that, although molecular weights of proteins to over 100,000 Da have been determined by ESI-quad analysis⁴, these materials have been essentially homogeneous. Problems arise in the analysis of proteins that are heterogeneous because of, for example, the presence of glycoforms. Such heterogeneity may not allow direct analysis of individual glycoforms because of lack of sufficient mass resolution with the quadrupole mass analyser. An average molecular weight of the heterogeneous mixture may be all that can be obtained by ESI-quad analysis, and then only if the complexity of the mixture does not over-

whelm the ability of the mass spectrometer to identify multiply-charged peaks.

Coupling of chromatography

The problem of analysing complex mixtures of closely-related materials such as glycoforms may be alleviated by partial separation of the mixture before performing ESI-quad analysis. This is an attractive option as ESI has been shown to be useful for the direct coupling of the mass spectrometer to HPLC⁶ and to HPCE⁷. An HPLC system with any column diameter can be coupled to the ESI-quad system, but as ESI is a low-liquid flow system operating optimally at 1–40 $\mu\text{l min}^{-1}$, larger column diameters require a solvent split after the column and before the mass spectrometer. To date, only a few HPLC solvent systems have been used with ESI and it remains to be seen how universal HPLC/MS coupling will be. Similarly, HPCE coupling to the ESI system has been demonstrated using, for example, a make-up liquid flow to increase the very low HPCE flow (< 1 $\mu\text{l min}^{-1}$) so that it is compatible with the ESI flow. Again, only limited buffer systems have been tested in such HPCE/MS couplings, and problems will undoubtedly arise with some buffers.

Structural analysis

Structural analysis of biomolecules, such as the sequence analysis of peptides, can be carried out using tandem mass spectrometry or MS/MS⁸. Using this approach, a protonated ion is generated from low-picomole amounts of the peptide of interest (even from a mixture of peptides) using the ionization technique of fast atom bombardment (FAB). The selected protonated ion is then forced to fragment by collision with a gas in a collision cell. The resulting sequence-specific fragment ions are then mass analysed in a second stage of mass analysis and interpreted in terms of the unknown peptide sequence. The same experiment can be carried out after ionization of the peptide by ESI instead of FAB ionization.

Several examples of MS/MS analysis of ESI-generated peptide ions have been reported⁴. While it is clear that the sequence-specific fragmentation obtained by this procedure is sufficient at least to confirm the sequence of known peptides, it is not yet clear that it will be routinely useful for the analysis of unknown peptide sequences. Difficulties arise, for example, when multiply-charged peptide parent ions fragment into multiply-charged fragment ions, and the charge state of the fragment ions is not apparent. Although development is at an early stage, a recent study demonstrated the fragmentation of a multiply-protonated protein ion generated by ESI, with subsequent MS/MS analysis of peptide fragments from the protein, resulting in sequence-specific

peptide fragment ions⁹. This is the first demonstration of peptide sequence ions being obtained directly from an intact protein in the gas phase.

Availability of ESI

Several manufacturers of mass spectrometers now offer ESI options for their quadrupole mass spectrometer systems. These are available as either single-stage mass analyser systems for molecular weight analysis, with the option of direct coupling to HPLC or HPCE systems, or as multistage analyser systems with the additional capability of structural analysis such as peptide sequencing by MS/MS.

Future prospects

Electrospray ionization has been shown to be useful for the molecular weight and structural analysis of carbohydrates and for the molecular weight analysis of small stretches of DNA (so far, to about 61 mer⁴). In addition, the technique holds considerable promise for high-sensitivity HPLC/MS and HPCE/MS analysis of many other biological compounds such as neurotransmitters and ionizable drug molecules.

Electrospray ionization should be useful for the high-sensitivity quantitative HPLC/MS and HPCE/MS analysis of peptides, proteins and other molecules by using the well-developed mass-spectrometric methods of selected ion monitoring along with stable isotopically-labelled internal standards.

When used with high-resolution mass spectrometers (for example, magnetic sector or Fourier transform ion cyclotron resonance systems), ESI should, in the future, allow complex mixture analysis, albeit at higher cost and greater instrument complexity. On the other hand, ESI may provide a possible route for the future development of routine, easy-to-use, low-cost HPLC/MS/MS and HPCE/MS/MS systems based on relatively inexpensive mass analysers, such as ion traps, for general analytical biochemistry. □

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Buyers' guide to Biotech '90

Macintosh-based sequence software, a trypsinization device for cells and a new line of preparative HPLC columns featuring a void-sealing mechanism are some of the highlights that can be seen at next week's Biotechnology '90 exhibition in Amsterdam, The Netherlands.

BOEHRINGER says its Glycan Differentiation Kit, which uses digoxigenin technology, can be used to **characterize carbohydrate moieties of glycoproteins** in as little as 4–5 hours (*Reader Service No. 101*). Highly specific and well-described lectins are conjugated to digoxigenin and used in combination with an alkaline phosphatase-conjugated anti-digoxigenin antibody; this provides a simple immunological detection method of bound lectins. The kit provides sufficient reagents for the analysis of terminal sugar residues on side chains of glycoproteins on 25 nitrocellulose filters (100 cm² each) using only 10–100-ng glycoprotein samples. Glycoprotein



Boehringer's glycan differentiation kit for rapid results in 4–5 hours.

researchers may be pleased to hear that glycoprotein samples do not have to be highly purified before use with the kit; this represents a considerable saving in time, says Boehringer. Drop by the Boehringer booth, number D101.

Researchers involved in studying the regulation of gene expression may be interested in Life Technologies' new series of **DNA binding protein detection systems** (*Reader Service No. 102*). Gibco-BRL brand AP-1, SP-1, NF- κ B and CRE binding protein detection systems detect the presence of DNA binding proteins using a gel retardation assay. The single-sequence, tandem-repeat oligonucleotide configuration is designed to provide increased sensitivity and improve interpretation of data. The detection systems contain specific enhancer oligonucleotide reagents for end labelling, positive control HeLa extracts, competitive DNA, and a set of instructions. Gibco's booth at Biotechnology '90 is D142.

Data handling

To meet the needs of molecular biologists, New Brunswick Scientific, exhibiting in booth D145, has a new **multipurpose densitometry system** called Sciden (*Reader Service No. 103*). Featuring a modular design, Sciden consists of a high-speed soft-laser scanner, video scanner



Sciden: NBS's multipurpose densitometry system has a modular design.

and monitor, IBM PC or compatible and accompanying software. The set-up can be used to sequence DNA, read ELISA and TLC plates, count colonies, size particles, and for image analysis. The high-speed laser scanner can take 1- and 2-D readings of autoradiograms and gels. Readings take about 30 minutes for a 2-D scan of a 20 × 20-cm gel or 12 seconds for a 1-D scan of a 20-cm lane, says NBS. The scanner can operate in reflectance, absorbance, transmittance or UV fluorescence mode; this makes Sciden as suitable for measuring fluorescent samples as for scanning pre-developed X-ray film or DNA sequences. Resolution with the laser scanner is 40 μ m and is independent of scan length, unlike the video scanner, which is limited to 50 μ m on 3 × 3-cm samples or up to 300 μ m for 20 × 20-cm plates. Scanning speed is, however, comparable. The software package includes 50 programs for specific applications, as well as standard operating routines and reporting methods.

Stop by the DNASTAR booth, number I114, and see the company's Mac-based **LaserGene sequence software package** (*Reader Service No. 104*). The LaserGene package includes £1,500 (UK) ProdiGene, which provides a powerful tool for analysing sequence data. Users can create restriction or plasmid maps, identify ORFs, translate DNA into protein, reverse translate protein into DNA sequences, label and annotate. Using 'filters' that partition enzymes based on class, site complexity, overhangs, or occurrence in vectors, it is possible to create complex subsets of restriction enzymes for the purposes of analysis or publication. Sequences can be entered via the keyboard, imported from 'foreign' software or entered from sequencing gels using DNASTAR's £2,500 (UK) SeqEasy II digitizer. ProdiGene is also available on compact disc (£2,500 (UK) ProdiGene-CD). In addition to the functions already

mentioned, the ProdiGene-CD package includes database access and analysis tools. Completing the line-up is £1,500 (UK) SeqMan — software for managing any sequencing task — and £92 (UK) GeneFont — a PostScript typeface designed for the molecular biologist.

Cell science

For the trypsinization of tissue cells before vaccine preparation and for other compounds that require non-destructive separation of tissue cells, Applikon recommends its **trypsinization apparatus** (*Reader Service No. 105*). The apparatus consists of a thick-walled glass cylinder



Applikon's trypsinization apparatus.

with stainless steel flange and special bottom plate that has a drain covered by a stainless steel wire screen. Fluid containing the trypsinized cells drains through the wire mesh and, in doing so, is effectively separated from the clumps and debris, says Applikon. A controlled vibrating action provides rapid trypsinization of the cells without damaging them. Optional extras include equipment that allows large cell volumes to be trypsinized and a heat exchanger for varying the temperature of the chamber. Applikon will be showing off the trypsinization apparatus together with new lines in bioreactors and control equipment for cell culture in booth D125.

Greiner's **tissue culture flask** product range now includes a Filter Cap T.C. flask that has a 0.2 μ micropore filter membrane built into the filter cap (*Reader Service No. 106*). According to Greiner, this arrangement provides contamination-free culture conditions, while allowing unrestricted gas exchange through the filter. Filter Cap T.C. flasks provide, says Greiner, optimum culture conditions for producing high cell yields by cutting down on evaporation losses and maintaining the pH. From cell scrapers to plasticware — Greiner's gadgets for cell culture will be in booth D131.

Sweet separation

Promega has introduced a new **reusable size-exclusion cartridge** called Rapid-Changer (*Reader Service No. 107*). The cartridges are designed for the rapid (two minute) desalting and buffer exchange of protein or nucleic acid samples and the

removal of unincorporated isotope or free nucleotides from protein or DNA labeling reactions. Two sizes are available for samples in the range of 50–200 μ l and 250 μ l–1 ml. RapidChanger cartridges provide 'tight' elution peaks and reproducible sample volumes, says Promega, making it easier for the user to judge when to begin and end sample collection. The cartridges can be autoclaved and reused up to twenty times. See Promega's products in booth D117.

Filtron sells a **centrifugal microconcentrator** — Microsep — that is designed for the rapid concentration, purification and desalting of small sample volumes of 3.5 ml or less (*Reader Service No. 108*).



Microsep: Filtron's small-volume centrifugal microconcentrators.

Microsep is suitable for use with biological samples containing peptides, proteins, enzymes, viruses, antibodies and other macromolecules. It can be used in one of two ways: by backspinning the concentrate into the concentrate cup of the device or by extracting the concentrate from the concentrate collector ring using a pipette tip. The device is sold preassembled and incorporates Filtron's Omega series low-protein binding PES ultrafiltration membrane. Membranes with molecular weight cut-offs of 3, 10, 30, 100, 300, and 1,000 kD are available. Microsep's design eliminates the O-ring; this, says Filtron, minimizes leachables and broadens the chemical compatibility range. Prices range between \$75 and 100 (US) for a pack of 24. Filtron's wares will be on show in booth D102.

Chromatography corner

For preparative HPLC methods development with scale up, Waters Chromatography Division of Millipore recommends the Delta Prep 4000 **HPLC system** (*Reader Service No. 109*). The Delta Prep 4000's wide flow-rate range (0.5 ml min⁻¹) allows methods development using analytical size columns with small volumes of sample and solvent, and then scale up to purify milligram-to-multigram quantities on the same system. As the system operates at pressures of up to 4,000 p.s.i. across the entire flow-rate range, all types of packing materials can be used, including 5 and 10 μ particle sizes in columns of up to 50-mm diameter or larger. Gradient



Waters' Delta Prep 4000 preparative HPLC system — see booth D132.

formation is made simple because the user has access to eleven pre-programmed gradient curves and can use up to four solvents: up to 15 gradient methods can be stored. See Waters in the Millipore booth, number D132.

Whatman Specialty Products Division is launching a new range of **preparative HPLC columns** featuring a void-sealing mechanism designed to eliminate the problems experienced during prolonged use when compression of the column bed results in the appearance of a void at the column head (*Reader Service No. 110*). Now, says Whatman, users can remove the void *in situ*, restoring chromatographic resolution and extending the column life without the need for repacking. The columns are available in four column diameters: 4.6, 9.5, 25.4 and 50.8 mm, in standard 25-mm lengths — although custom lengths are available on request.



Preparative HPLC columns from Whatman with void-sealing mechanism.

Users can choose column packings from the Whatman Partisil BioPrep silica media range: 10 and 20 μ m, normal and reverse phase, with a choice of three pore sizes — 75, 150 and 300 Å are available. Columns can be supplied empty or, for convenience, pre-packed. Watch out for Whatman in Amsterdam in booth D141. □

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